

**GAPPED OLIGOMERS HAVING SITE SPECIFIC CHIRAL  
PHOSPHOROTHIOATE INTERNUCLEOSIDE LINKAGES**

**CROSS REFERENCE TO RELATED APPLICATIONS**

This patent application is a continuation-in-part of Application Serial No. 09/325,058, filed on July 14, 1999, entitled "Oligonucleotides Having Site Specific Chiral Phosphorothioate Internucleoside Linkages", the contents of which is incorporated herein by reference in its entirety.

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**FIELD OF THE INVENTION**

This invention relates to the design and synthesis of nuclease resistant gapped oligonucleotides which are useful for therapeutics, diagnostics and as research reagents.

15 Oligomers are provided having internal deoxyribose regions having all chiral Rp phosphorothioate internucleoside linkages and external regions having nuclease resistant modifications. Such oligomers are resistant to nuclease degradation and are capable of modulating the activity of  
20 DNA and RNA.

**BACKGROUND OF THE INVENTION**

It is well known that most of the bodily states in multicellular organisms, including most disease states, are effected by proteins. Such proteins, either acting directly

or through their enzymatic or other functions, contribute in major proportion to many diseases and regulatory functions in animals and man. For disease states, classical therapeutics has generally focused upon interactions with  
5 such proteins in efforts to moderate their disease-causing or disease-potentiating functions. In newer therapeutic approaches, modulation of the actual production of such proteins is desired. By interfering with the production of proteins, the maximum therapeutic effect can be obtained  
10 with minimal side effects. It is therefore a general object of such therapeutic approaches to interfere with or otherwise modulate gene expression, which would lead to undesired protein formation.

One method for inhibiting specific gene expression is  
15 with the use of oligonucleotides, especially oligonucleotides which are complementary to a specific target messenger RNA (mRNA) sequence. Several oligonucleotides are currently undergoing clinical trials for such use. Phosphorothioate oligonucleotides are  
20 presently being used as therapeutic agents in human clinical trials against various disease states, including use as antiviral agents.

In addition to such use as both indirect and direct regulators of proteins, oligonucleotides also have found use  
25 in diagnostic tests. Such diagnostic tests can be performed using biological fluids, tissues, intact cells or isolated cellular components. As with gene expression inhibition, diagnostic applications utilize the ability of oligonucleotides to hybridize with a complementary strand of  
30 nucleic acid. Hybridization is the sequence specific hydrogen bonding of oligomeric compounds via Watson-Crick and/or Hoogsteen base pairs to RNA or DNA. The bases of such base pairs are said to be complementary to one another.

Oligonucleotides are also widely used as research  
35 reagents. They are useful for understanding the function of

many other biological molecules as well as in the preparation of other biological molecules. For example, the use of oligonucleotides as primers in PCR reactions has given rise to an expanding commercial industry. PCR has  
5 become a mainstay of commercial and research laboratories, and applications of PCR have multiplied. For example, PCR technology now finds use in the fields of forensics, paleontology, evolutionary studies and genetic counseling. Commercialization has led to the development of kits which  
10 assist non-molecular biology-trained personnel in applying PCR. Oligonucleotides, both natural and synthetic, are employed as primers in such PCR technology.

Oligonucleotides are also used in other laboratory procedures. Several of these uses are described in common  
15 laboratory manuals such as *Molecular Cloning, A Laboratory Manual*, Second Ed., J. Sambrook, et al., Eds., Cold Spring Harbor Laboratory Press, 1989; and *Current Protocols In Molecular Biology*, F. M. Ausubel, et al., Eds., Current Publications, 1993. Such uses include as synthetic  
20 oligonucleotide probes, in screening expression libraries with antibodies and oligomeric compounds, DNA sequencing, *in vitro* amplification of DNA by the polymerase chain reaction, and in site-directed mutagenesis of cloned DNA. See Book 2 of *Molecular Cloning, A Laboratory Manual, supra*. See also  
25 "DNA-protein interactions and The Polymerase Chain Reaction" in Vol. 2 of *Current Protocols In Molecular Biology, supra*.

A number of chemical modifications have been introduced into oligonucleotides to increase their usefulness in diagnostics, as research reagents and as  
30 therapeutic entities. Such modifications include those designed to increase binding to a target strand (i.e. increase melting temperatures,  $T_m$ ), to assist in identification of an oligonucleotide or an oligonucleotide-target complex, to increase cell penetration, to stabilize

against nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotides, to provide a mode of disruption (terminating event) once sequence-specifically bound to a target, and to improve the pharmacokinetic properties of the oligonucleotide.

The complementarity of oligonucleotides has been used for inhibition of a number of cellular targets. Complementary oligonucleotides are commonly described as being antisense oligonucleotides. Various reviews describing the results of these studies have been published including Progress In Antisense Oligonucleotide Therapeutics, Crooke, S.T. and Bennett, C.F., *Annu. Rev. Pharmacol. Toxicol.*, **1996**, 36, 107-129. These oligonucleotides have proven to be powerful research tools and diagnostic agents. Certain oligonucleotides that have been shown to be efficacious are currently in human clinical trials.

The pharmacological activity of oligonucleotides, like other therapeutics, depends on a number of factors that influence the effective concentration of these agents at specific intracellular targets. One important factor for oligonucleotides is the stability of the species in the presence of nucleases. It is unlikely that unmodified, naturally-occurring oligonucleotides will be useful therapeutic agents because they are rapidly degraded by nucleases. The limitations of available methods for modification of the phosphate backbone of unmodified oligonucleotides have led to a continuing and long felt need for other modifications which provide resistance to nucleases and satisfactory hybridization properties for antisense oligonucleotide diagnostics and therapeutics.

Oligonucleotides having phosphorothioate modified backbones have shown therapeutic effects against numerous targets. This success is due in part to the increased

A specific feature of oligonucleotides as drugs is that they must be stable *in vivo* long enough to be effective. Consequently, much research has been focused on enhancing the stability of oligonucleotide therapeutics while maintaining their specific binding properties. Recently, several groups have reported that chiral phosphorothioate oligonucleotide analogs have enhanced binding properties (Rp isomer) to the target RNA as well as significant stabilization to exonucleases (Sp isomer) (See Koziolkiewicz *et al.*, *Antisense & Nucleic acid drug development*, **1997**, 7, 43-8; Burgers *et al.*, *J. Biol. Chem.*, **1979**, 254, 6889-93; and Griffiths *et al.*, *Nucleic Acids Research*, **1987**, 15, 4145-62).

Presently, there is no method to prepare P-chiral oligonucleotides in large scale. Current methods include synthesis and chromatographic isolation of stereoisomers of the chiral building blocks. (Stec et al., *Angew. Chem. Int. Ed. Engl.*, **1994**, 33, 709; Stec et al., *J. Am. Chem. Soc.*, **1995**, 117, 12019; and Stec WJ., *Protocols for Oligonucleotides and Analogs: Synthesis and Properties*, edited by Sudhir Agrawal, p. 63-80, (1993, Humana Press) and references

cited therein). This method suffers from the non-stereospecific synthesis of the synthon. Recently, Just and coworkers presented the use of a chiral auxiliary to form dinucleotide phosphorothioate triesters in 97%ee (Wang, JC.,  
5 and Just G., *Tetrahedron Letters*, **1997**, 38, 705-708).

However, there was reported difficulty in removing the chiral auxiliary protecting group at phosphorous. This method has yet to be tested for convenient large scale automated synthesis.

10 Stereoregular phosphorothioate analogs of pentadecamer 5'-d(AGATGTTTGA GCTCT)-3' were synthesized by the oxathiaphospholane method (Koziolekiewicz *et al.*, *Nucleic Acids Res.*, **1995**, 23, 5000-5005). Their diastereomeric  
15 purity was assigned by means of enzymic degradation with nuclease P1 and independently, with snake venom phosphodiesterase. DNA-RNA hybrids formed by phosphorothioate oligonucleotides (PS-oligos) with the corresponding complementary pentadecaribonucleotide were treated with bacterial RNase H. The DNA-RNA complex  
20 containing the all Rp phosphorothioate oligomer was found to be more susceptible to RNase H-dependent degradation of the pentadecaribonucleotide compared with hybrids containing either the [all-SP] counterpart or the so called random mixture of diastereomers of the pentadeca(nucleoside  
25 phosphorothioate). This stereodependence of RNase H action was also observed for a polyribonucleotide (475 nt) hybridized with these phosphorothioate oligonucleotides. The results of melting studies of PS-oligo-RNA hybrids allowed a rationalization of the observed stereo-  
30 differentiation in terms of the higher stability of heterodimers formed between oligoribonucleotides and [all-Rp]-oligo(nucleoside phosphorothioates), compared with the less stable heterodimers formed with [all-Sp]-oligo-  
(nucleoside phosphorothioates) or the random mixture of  
35 diastereomers.

(S)-1-(indol-2-yl)-propan-2-ol was used as a chiral auxiliary to form a dinucleotide phosphorothioate triester in 97% ee (Wang et al., *Tetrahedron Lett.*, **1997**, 38, 705-708).

5 A stereoselective preparation of dinucleotide phosphorothioates with a diastereomeric excess of >98%, using hydroxy(indolyl)butyronitrile I as chiral auxiliaries, is reported (Wang et al., *Tetrahedron Lett.*, **1997**, 38, 3797-3800).

10 1,2-O-Cyclopentylidene-5-deoxy-5-isopropylamino-D-xylofuranose and its enantiomer were used as chiral auxiliaries to form, respectively, Sp and Rp dithymidine phosphorothioates in 98% diastereomeric excess, using phosphoramidite methodologies and 2-bromo-4,5-  
15 dicyanoimidazole as catalyst (Jin et al., *J. Org. Chem.*, **1998**, 63, 3647-3654).

Oligonucleotide phosphorothioates were synthesized using prokaryotic DNA polymerase and oligonucleotide template/primer (Lackey et al., *Biotechnol. Lett.*, **1997**, 19,  
20 475-478). The method facilitates the recovery of DNA polymerase and template/primer and is successful at the milligram scale. Thus, reusable template/primers were designed to specify the synthesis of an oligonucleotide (GPs0193) complementary to a sequence in exon 7 of the human  
25 immunodeficiency virus genome. Extension of the 3'-terminus by DNA polymerase utilizing dNTPS(Rp+Sp) substrates produced the specified oligonucleotide phosphorothioate with the chirally pure (Rp) stereochemistry. The biochemical synthesis was essentially complete within 60 minutes  
30 (compared with 24 h for automated solid phase synthesis), and produced <5% intermediate length oligonucleotide products, corresponding to a stepwise yield of >99.7% for the addition of each nucleotide.

- Phosphorothioate oligodeoxyribonucleotides were tested for their ability to recognize double-helical DNA in two distinct triple helix motifs (Hacia et al., *Biochemistry*, **1994**, 33, 5367-5369). Purine-rich oligonucleotides
- 5 containing a diastereomeric mixture of phosphorothioate or stereoregular (all Rp) phosphorothioate linkages are shown to form triple-helical complexes with affinities similar to those of the corresponding natural phosphodiester oligonucleotides. In contrast, pyrimidine-rich
- 10 phosphorothioate oligonucleotides containing a mixture of diastereomeric or stereoregular (all Rp) linkages do not bind to double-helical DNA with measurable affinity. These observations have implications for triple helix structure and for biological applications.
- 15 An enzymatic protocol has been established for the synthesis of stereoregular (all Rp) oligodeoxyribonucleotide phosphorothioates. A 25-mer oligodeoxynucleotide phosphorothioate has been synthesized and studied for biophysical and biochemical properties (Tang et al.,
- 20 *Nucleosides and Nucleotides*, **1995**, 14, 985-990).
- Stability of oligo(nucleoside phosphorothioate)s (PS-oligos) in HUVEC (human umbilical vein endothelial cells) has been studied (Koziolkiewicz et al., *Nucleosides and Nucleotides*, **1997**, 16, 1677-1682). Cytosolic fraction of
- 25 HUVEC possesses 3'-exo-nucleolytic activity which is responsible for degradation of natural and PS-oligomers. The enzyme is Rp-specific, i.e. it cleaves internucleotide phosphorothioate function of Rp- and not Sp-configuration at phosphorus atom.
- 30 Enzymatic hydrolysis of stereoregular oligodeoxyribonucleoside phosphorothioates (PS-oligos) synthesized via the oxathiaphospholane method has been used for assignment of their diastereomeric purity (Koziolkiewicz et al., *Antisense Nucleic Acid Drug Dev.*, **1999**, 9, 171-181). For

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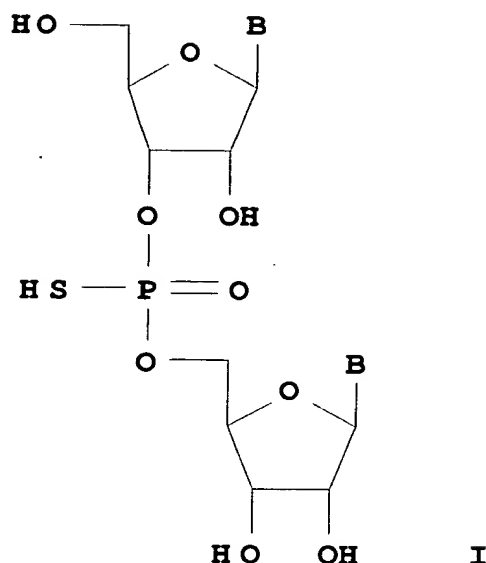
5 this purpose, two well-known enzymes of established  
diastereoselectivity, nuclease P1 and snake venom phospho-  
diesterase (svPDE) have been used. However, because of some  
disadvantageous properties of svPDE, a search for other  
10 [Rp]-specific endonucleases was undertaken. Extracellular  
bacterial endonuclease isolated from *Serratia marcescens*  
accepts PS-oligos as substrates and hydrolyzes  
phosphorothioate bonds of the [Rp] configuration, whereas  
internucleotide [Sp]-phosphorothioates are resistant to its  
15 action. Cleavage experiments carried out with the use of  
unmodified and phosphorothioate oligonucleotides of  
different sequences demonstrate that the *Serratia* nuclease  
is more selective in recognition and hydrolysis of  
oligodeoxyribonucleotides than previously reported. The  
20 substrate specificity exhibited by the enzyme is influenced  
not only by the nucleotide sequence at the cleavage site but  
also by the length and base sequence of flanking sequences.  
The *Serratia* nuclease can be useful for analysis of  
diastereomeric purity of stereodefined phosphorothioate  
oligonucleotides, but because of its sequence preferences,  
the use of this enzyme in conjunction with svPDE is more  
reliable.

An NMR study of the structure of dissolved DNA/RNA  
hybrid containing stereoregular Rp-phosphorothioate  
25 modifications of all DNA backbone linkages was recently  
examined. The complex of the enzymatically synthesized  
phosphorothioate DNA octamer (all-Rp)-d(GCGTCAGG) and its  
complementary RNA r(CCUGACGC) had an overall conformation  
within the A-form family (Bachelin et al., *Nat. Struct.*  
30 *Biol.*, **1998**, 5, 271-276). Most helical parameters and the  
sugar puckers of the DNA strand assume values intermediate  
between A- and B-form. The close structural similarity with  
the unmodified DNA/RNA hybrid of the same sequence may  
explain why both the natural and the sulfur-substituted  
35 complex can be recognized and digested by RNase H.

New monomers, 5'-O-DMT-deoxyribonucleoside 3'-O-(2-thio-"spiro"-4,4-penta-methylene-1,3,2-oxathiaphospholane)s, were prepared and used for the stereo-controlled synthesis of PS-Oligos via the oxathiaphospholane approach (Stec et al., *J. Am. Chem. Soc.*, **1998**, 120, 7156-7167). These monomers and their 2-oxo analogs were used for the synthesis of "chimeric" constructs (PS/PO-oligos) possessing phosphate and P-stereo-defined phosphorothioate inter-nucleotide linkages. The yield of a single coupling step is approximately 92-95%, and resulting oligomers are free of nucleobase- and sugar-phosphorothioate backbone modifications. Thermal dissociation studies showed that for hetero-duplexes formed by [Rp]-, [Sp]-, or [mix]-PS/PO-T10 with dA12, dA30, or poly(dA), for each template, the melting temperatures as well as free Gibbs' energies of dissociation process, are virtually equivalent. Stereochemical evidence derived from crystallographic analysis of one of the oxathiaphospholane monomers strongly supports the participation of pentacoordinate intermediates in the mechanism of the oxathiaphospholane ring-opening condensation.

The DBU-assisted 1,3,2-oxathiaphospholane ring opening condensation of the separate diastereomers of 5'-O-DMT-2'-O-TBDMS-ribonucleoside-3'-O-(2-thiono-1,3,2-oxathiaphospholane)s with 2'-TBDMSi-protected ribonucleoside bound to the solid support via the 3'-oxygen occurs with 96-100% stereospecificity and gives, after deprotection, [Rp]- or [SP]-diribonucleoside 3',5'-phosphorothioates I (B = adenine, cytosine, guanine, uracil) in 65-97% yield (Sierzcha-la et al., *J. Org. Chem.*, **1996**, 61, 6713-6716). Attempts to improve these yields by increasing either the coupling time or DBU concentration were unsuccessful. The absolute configuration at phosphorus of the dimers (I) was assigned by treatment with the stereospecific nucleases svPDE or nuclease P1. Discrimination of [Rp]- vs [Sp]- diastereomers of the following dimer by nuclease P1 is much

less profound than that observed for dideoxyribonucleoside 3',5'-phosphorothioates.



Diastereomerically pure 5'-O-DMT-nucleoside 3'-O-(2-thio-1,3,2-oxathiaphospholanes) (B = T, Adebz, Cytbz) were used for the synthesis of stereo-regular phosphorothioates (Stec et al., *J. Am. Chem. Soc.*, **1995**, 117, 12019-12029). The oxathiaphospholane ring-opening condensation requires the presence of strong organic base, preferably DBU. The yield of a single coupling step is ca. 95% and resulting S-oligos are free of nucleobase- and sugar-phosphorothioate backbone modifications. The diastereomeric purity of products was estimated on the basis of diastereoselective degradation with Nuclease P1 and a mixture of svPDE and *Serratia marcescens* endonuclease. Thermal dissociation studies of hetero-duplexes phosphorothioates/DNA and phosphorothioates/RNA showed that their stability is stereochemical- and sequence-dependent.

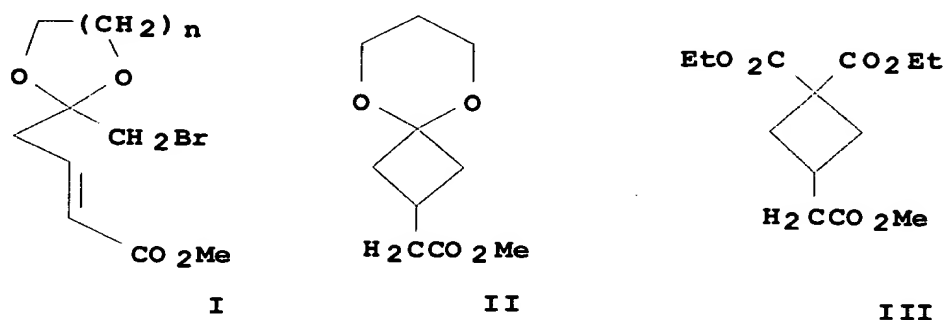
It has been previously reported that four membered cyclic sulfur compounds are kinetically and thermodynamically facile compounds to form (Eliel et al., *J. Am. Chem.*

Soc., 1985, 107, 2946-2952). A combination of product and rate studies including Hammett LFER for  $k$  and  $k_s$  for  $p$ -substituted 3-(arylthio)-3-methyl-1-Bu tosylates and the solvent and salt effects on product ratio indicate that anchimeric assistance in the solvolysis of branched 3-(alkylthio) and (3-arylthio)propyl tosylates is real and that a marked Thorpe-Ingold effect is evident. This observation led to the design of compounds shown in Figures 2 to 7 as chiral auxiliaries to synthesize chiral phosphorothioates.

In a similar publication the neighboring group participation of oxygen in the solvolysis of acyclic-alkoxy substituted  $p$ -toluenesulfonates was illustrated (Elie<sup>l</sup> et al., *J. Org. Chem*, 1985, 50, 2707-2711). Methanolysis of  $\text{PhCH}_2\text{OCRR}_1\text{CR}_2\text{R}_3\text{CHR}_4\text{OTs}$  ( $\text{R} = \text{Me}$ ,  $\text{R}_1\text{-R}_4 = \text{H}$ ;  $\text{R} = \text{R}_1 = \text{Me}$ ,  $\text{R}_2\text{-R}_4 = \text{H}$ ;  $\text{R} = \text{R}_1 = \text{R}_4 = \text{Me}$ ,  $\text{R}_2 = \text{R}_3 = \text{H}$ ;  $\text{R} = \text{R}_1 = \text{R}_3 = \text{R}_4 = \text{H}$ ,  $\text{R}_2 = \text{Me}$ ;  $\text{R} = \text{R}_1 = \text{R}_4 = \text{H}$ ,  $\text{R}_2 = \text{R}_3 = \text{Me}$ ;  $\text{Ts} = \text{O}_2\text{SC}_6\text{H}_4\text{Me-}p$ ) proceeds with partial rearrangement, implying neighboring-group participation, only when there are geminal Me groups in the 2- or 3-position ( $\text{R}_2 = \text{R}_3 = \text{Me}$  or  $\text{R} = \text{R}_1 = \text{Me}$ ).

In a recent review article entitled "New gem- and vic-disubstituent effects on cyclizations", (Jung, Michael E., *Synlett*, 1999, 843-846 a summary of several new gem-disubstituent effects on cyclizations are illustrated, e.g., the gem-dialkoxy, -dicarboalkoxy, and -dithioalkoxy effects, have been discovered. In addition they have also observed a new vicinal disubstituent effect. A novel ring size effect of ketals on radical cyclizations has been investigated. In a similar article by the same author it was disclosed that while reaction of the bromoalkene with a 5-membered ketal I ( $\text{R} = \text{Br}$ ,  $n = 1$ ) with tributyltin hydride gave only the acyclic product I ( $\text{R} = \text{H}$ ,  $n = 1$ ), reaction of the corresponding bromoalkene with a 6-membered ketal I ( $\text{R} = \text{Br}$ ,  $N = 2$ ) gave good yields of the cyclobutane II, in a novel ketal ring size effect. Also the gem-dicarboalkoxy effect

was operative in these systems, e.g., cyclization of the bromo alkene triester, (E)-MeO<sub>2</sub>CCH:CHCH<sub>2</sub>C(CO<sub>2</sub>Et)<sub>2</sub>CH<sub>2</sub>OC(:S)OPh, afforded reasonable yields of the cyclobutane III.



5 In accordance with this theory, the structures 3, 8, 14, 18, 20, and 25 all have geminal disubstituents. Use of this concept to synthesize chiral phosphorothioates with the concurrent formation of 4-membered cyclic thio compounds is novel.

10 Oligonucleotides that have chiral Sp phosphorothioate internucleotide linkages at the 3'-terminus are disclosed in International Publication Number WO 99/05160, published February 4, 1999.

Methods for the enzymatic synthesis of oligonucleo-  
15 tides are disclosed by Hyman in United States Patent Serial No., 5,602,000 issued February 11, 1997, entitled "Method for Enzymatic Synthesis of Oligonucleotides".

The solid-phase stereoselective synthesis of 2'-O-methyl oligoribonucleoside phosphorothioates has been  
20 reported using bicyclic oxazaphospholidines (Guo et al., *Bioorganic & Medicinal Chemistry Letters*, 1998, 8, 2539-2544).

Enzymes are also being used to prepare random  
libraries (aptamers) having both phosphodiester and  
25 phosphorothioate internucleoside linkages where the

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phosphorothioate internucleoside linkages are chiral Rp linkages (King *et al.*, *Biochemistry*, 1998, 37, 16489-16493).

# **SUMMARY OF THE INVENTION**

5       The present invention provides nuclease resistant oligomeric compounds which are useful for therapeutics, diagnostics and as research reagents.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the structure of an Rp and an Sp chiral  
10 phosphorothioate internucleotide linkage.

Figure 2 shows the chiral adjuvant (R)-4-mercapto-4-methyl-2-pentanol and the chiral building block derived therefrom which leads to Rp chiral phosphorothioate internucleotide linkages.

15       Figure 3 shows the chiral adjuvant (S)-4-mercapto-4-methyl-2-pentanol and the chiral building block derived therefrom which leads to Sp chiral phosphorothioate internucleotide linkages.

Figure 4 shows (+)-5-methyl-2-(1-methyl-1-thioethyl) cyclohexanol, which is obtained from (+)-pulegone, and the chiral building block derived therefrom which leads to Rp chiral phosphorothioate internucleotide linkages.

Figure 5 shows (-)-5-methyl-2-(1-methyl-1-thioethyl) cyclohexanol, which is obtained from (-)-pulegone, and the  
25 chiral building block derived therefrom which leads to Sp chiral phosphorothioate internucleotide linkages.

Figure 6 shows 5C-methyl-2t-[(1-methyl-1-benzylamino)ethyl]-cyclohexanol which is obtained from (+)-pulegone, and the chiral building block derived therefrom  
30 which leads to Rp chiral phosphorothioate internucleotide linkages.

Figure 7 shows 5C-methyl-2t-[(1-methyl-1-benzylamino)ethyl]-cyclohexanol which is obtained from (-)-

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pulegone, and the chiral building block derive therefrom which leads to Sp chiral phosphorothioate internucleotide linkages.

Figure 8 shows monomeric and dimeric building blocks that are added to the 5'-end of a gapped oligomeric compound.

Figure 9 shows monomeric and dimeric building blocks that are added to the 3'-end of a gapped oligomeric compound.

Figure 10 shows procedures and products formed when a 5'-building block is added to a middle section and further coupling processes to form the internal region.

Figure 11 shows procedures and products formed when a 5'-building block is added to a middle section and further coupling processes to form the internal region.

Figure 12 shows procedures and products formed when a  $Pg_2$  group is removed from a 3'-end of a completed internal region with the addition of a further nucleomonomer to complete the gapped oligomer.

Figure 13 shows coupling of 3'-pieces to completed internal regions that also have completed 5'-ends (5'-external region).

Figure 14 shows gapped oligomeric compounds after the removal of  $Pg_1$ .

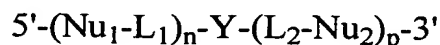
Figure 15 shows gapped oligomeric compounds after the removal of  $Pg_1$ .

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides gapped oligomeric compounds (gapmers) that have improved binding affinity and nuclease resistance relative to unmodified oligonucleotides. The gapmers are prepared having an internal chiral Rp deoxy-phosphorothioate region flanked on each end by external regions. The internal region imparts enhanced binding affinity to targets such as complementary nucleic acids.

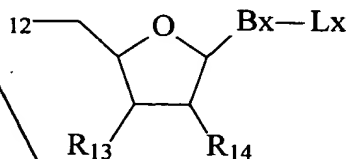
The external regions of linked nucleosides are altered to impart enhanced nuclease resistance to the gapmers. Such alterations include modifying the points of attachment or the chemistry of the internucleotide linkage, the addition of substituent groups and the modification of sugar configurations.

In a preferred embodiment the oligomeric compounds of the invention have the formula:



10 wherein:

each  $Nu_1$  and  $Nu_2$ , independently, has the formula:



wherein

Bx is a heterocyclic base moiety;

Lx is hydrogen, a protecting group or a substituent group;

15

one of  $R_{12}$ ,  $R_{13}$  and  $R_{14}$  is hydroxyl, a protected hydroxyl, a covalent attachment to a solid support, a nucleoside, an oligonucleoside, a nucleotide, an oligonucleotide, a conjugate group or an optionally

20 protected substituent group;

another of  $R_{12}$ ,  $R_{13}$  and  $R_{14}$  is hydrogen, hydroxyl, a protected hydroxyl or an optionally protected substituent group;

the remaining of  $R_{12}$ ,  $R_{13}$  and  $R_{14}$  of  $Nu_1$ , is  $L_1$ ;

25 the remaining of  $R_{12}$ ,  $R_{13}$  and  $R_{14}$  of  $Nu_2$ , is  $L_2$ ;

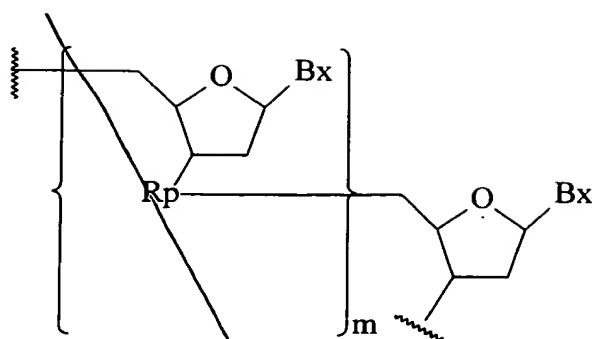
each  $L_1$  and each  $L_2$  is, independently, a phosphodiester internucleoside linkage or a modified internucleoside linkage;

Y has the formula:

Subt  
A2

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wherein:

each Rp is a chiral Rp phosphorothioate internucleotide linkage; and

each n, m and p is, independently, from 1 to 100; where  
5 the sum of n, m and p is from 3 to about 200;

with the proviso that at least one of R<sub>12</sub>, R<sub>13</sub>, R<sub>14</sub> and L<sub>x</sub> is a substituent group or at least one of L<sub>1</sub> and L<sub>2</sub> is a modified internucleoside linkage.

In one aspect of the present invention internucleoside  
10 linkages of one or both of the external regions are modified to enhance the nuclease resistance of the resulting oligomeric compound. Essentially all of the internucleoside linkages of the external regions could be modified in such a manner. In a preferred embodiment both of the external regions comprise a  
15 single nucleoside and a single modified internucleoside linkage bound to the internal region. Included are chemical modifications, positional modifications or combinations of both.

Representative phosphorus and non-phosphorus containing  
20 internucleoside linking moieties that impart nuclease resistance are well documented in the prior art and include without limitation the following:

phosphorus containing linkages

- phosphorodithioate (-O-P(S)(S)-O-);
- 25 Sp phosphorothioate (-O-P(S)(O)-O-);
- phosphoramidate (-O-P(O)(NJ)-O-);
- thiophosphoramidate (-O-P(O)(NJ)-S-);

- phosphonate  $(-O-P(J)(O)-O-)$ ;  
methylene phosphonate  $(-CH_2-P(O)(O)-O-)$ ;  
phosphotriesters  $(-O-P(O)(J)(O)-O-)$ ;  
thionoalkylphosphonate  $(-O-P(S)(J)-O-)$ ;  
5 thionoalkylphosphotriester  $(-O-P(O)(OJ)-S-)$ ;  
boranophosphate  $(-O-P(O)(BJ_3)-J-)$ ;  
boranothiophosphate  $(-O-P(S)(BJ_3)-J-)$ ;  
non-phosphorus containing linkages  
thiodiester  $(-O-C(O)-S-)$ ;  
10 thionocarbamate  $(-O-C(O)(NJ)-S-)$ ;  
siloxane  $(-O-Si(J)_2-O-)$ ;  
carbamate  $(-O-C(O)-NH-$  and  $-NH-C(O)-O-)$   
sulfamate  $(-O-S(O)(O)-N-$  and  $-N-S(O)(O)-N-$ ;  
morpholino sulfamide  $(-O-S(O)(N(morpholino))-)$ ;  
15 sulfonamide  $(-O-SO_2-NH-)$ ;  
sulfide  $(-CH_2-S-CH_2-)$ ;  
sulfonate  $(-O-SO_2-CH_2-)$ ;  
 $N,N'$ -dimethylhydrazine  $(-CH_2-N(CH_3)-N(CH_3)-)$ ;  
thioformacetal  $(-S-CH_2-O-)$ ;  
20 formacetal  $(-O-CH_2-O-)$ ;  
thioketal  $(-S-C(J)_2-O-)$ ; and  
ketal  $(-O-C(J)_2-O-)$ ;  
amine  $(-NH-CH_2-CH_2-)$ ;  
hydroxylamine  $(-CH_2-N(J)-O-)$ ;  
25 hydroxyimine  $(-CH=N-O-)$ ; and  
hydrazinyl  $(-CH_2-N(H)-N(H)-)$ .

"J" denotes a substituent group which is commonly hydrogen or an alkyl group, but which can be a more complicated group that varies from one type of linkage to  
30 another.

In addition to linking groups as described above that involve the modification or substitution of one or more of the  $-O-P(O)_2-O-$  atoms of a naturally occurring linkage, included within the scope of the present invention are linking groups  
35 that include modification of the 5'-methylene group as well as



5 The use of one or more L-ribose sugars in the external regions is also amenable to the present invention (Damha M.J., *et al.*, **1994**, 33, 7877-7885). The disclosures of each of the above referenced publications are herein incorporated by reference.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleoside linkages that do not include a phosphorus atom therein include alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or  
35 cycloalkyl internucleoside linkages, or one or more short

chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thio-  
5 formacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

10 Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307;  
15 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,633,360; 5,663,312; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

20 Positional modifications, also known in the art, involve the linking of nucleosides in a non-naturally occurring motif. As used herein the term "positional modification" is meant to include without limitation 2',5'-internucleoside linkages. Combining modifications e.g. using modified chemistries and  
25 positional modifications of selected internucleoside linkages is also amenable to the present invention where for example a 2',5'-phosphoramidate internucleoside linkage is employed. The 2'-5'-linkage has been used at the termini of oligomeric compounds to enhance the nuclease resistance (as described in  
30 U.S. Application Serial No. 09/115,043, filed July 14, 1998).

In another aspect of the present invention nuclease resistance is imparted to oligomeric compounds by covalently attaching a substituent group to at least one nucleoside in one or both of the external regions. In one embodiment each  
35 of the nucleosides in the external regions have a covalently

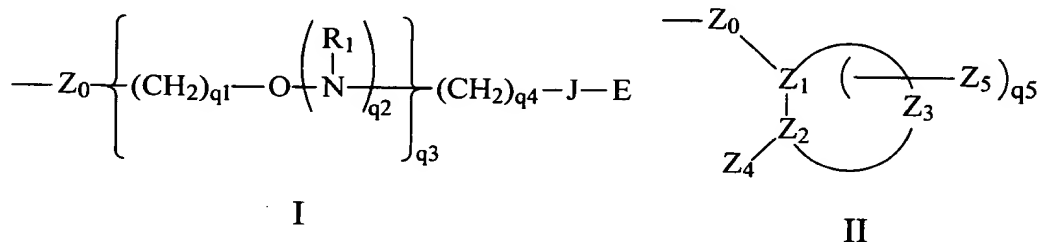
attached substituent group. Generally, selected nucleosides will have a substituent group covalently attached to a 2', 3' or 5'-position of a sugar moiety. In addition to the sugar moiety a heterocyclic base moiety can also have a substituent group attached thereto. Substituent groups can be covalently attached to purines at the N2 or N6 position and pyrimidines at the N4 or C5 position. A preferred position is the 2' position of the sugar moiety.

A representative list of substituent groups amenable to the present invention include C<sub>1</sub>-C<sub>20</sub> alkyl, C<sub>2</sub>-C<sub>20</sub> alkenyl, C<sub>2</sub>-C<sub>20</sub> alkynyl, C<sub>5</sub>-C<sub>20</sub> aryl, O-alkyl, O-alkenyl, O-alkynyl, O-alkylamino, O-alkylalkoxy, O-alkylaminoalkyl, O-alkyl imidazole, S-alkenyl, S-alkynyl, NH-alkyl, NH-alkenyl, NH-alkynyl, N-dialkyl, O-aryl, S-aryl, NH-aryl, O-aralkyl, S-aralkyl, NH-aralkyl, N-phthalimido, halogen (particularly fluoro), keto, carboxyl, nitro, nitroso, nitrile, trifluoromethyl, trifluoromethoxy, imidazole, azido, hydrazino, hydroxylamino, isocyanato, sulfoxide, sulfone, sulfide, disulfide, silyl, heterocycle, carbocycle, polyamine, polyamide, polyalkylene glycol, and polyethers of the formula (O-alkyl)<sub>m</sub>, where m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi et al. (*Drug Design and Discovery* **1992**, 9, 93), Ravasio et al. (*J. Org. Chem.* **1991**, 56, 4329) and Delgado et al. (*Critical Reviews in Therapeutic Drug Carrier Systems* **1992**, 9, 249), each of which is herein incorporated by reference in its entirety. Further sugar modifications are disclosed in Cook, P.D., *Anti-Cancer Drug Design*, **1991**, 6, 585-607. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl amino substitution is described in United States Patent Application serial number 08/398,901, filed March 6, 1995, entitled Oligomeric Compounds having Pyrimidine Nucleotide(s) with 2'

and 5' Substitutions, hereby incorporated by reference in its entirety.

Additional substituent groups amenable to the present invention include -SR and -NR<sub>2</sub> groups, wherein each R is, independently, hydrogen, a protecting group or substituted or unsubstituted alkyl, alkenyl, or alkynyl. 2'-SR nucleosides are disclosed in United States Patent No. 5,670,633, issued September 23, 1997, hereby incorporated by reference in its entirety. The incorporation of 2'-SR monomer synthons are disclosed by Hamm et al., *J. Org. Chem.*, **1997**, 62, 3415-3420. 2'-NR<sub>2</sub> nucleosides are disclosed by Goettingen, M., *J. Org. Chem.*, **1996**, 61, 6273-6281; and Polushin et al., *Tetrahedron Lett.*, **1996**, 37, 3227-3230.

Further substituent groups have one of formula I or II:

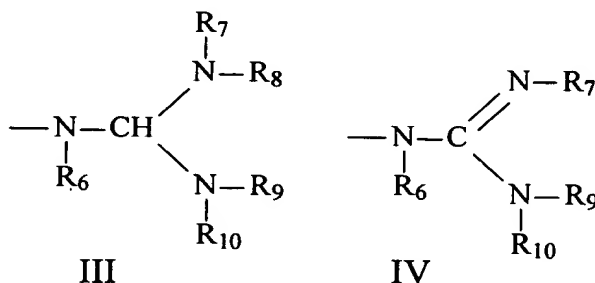


15 wherein:

Z<sub>0</sub> is O, S or NH;

J is a single bond, O or C(=O);

E is C<sub>1</sub>-C<sub>10</sub> alkyl, N(R<sub>1</sub>)(R<sub>2</sub>), N(R<sub>1</sub>)(R<sub>5</sub>), N=C(R<sub>1</sub>)(R<sub>2</sub>), N=C(R<sub>1</sub>)(R<sub>5</sub>) or has one of formula III or IV;



20 each R<sub>6</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub> and R<sub>10</sub> is, independently, hydrogen, C(O)R<sub>11</sub>, substituted or unsubstituted C<sub>1</sub>-C<sub>10</sub> alkyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkenyl, substituted or unsubstituted

C<sub>2</sub>-C<sub>10</sub> alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl,

5 aryl, alkenyl and alkynyl;

or optionally, R<sub>7</sub> and R<sub>8</sub>, together form a phthalimido moiety with the nitrogen atom to which they are attached;

or optionally, R<sub>9</sub> and R<sub>10</sub>, together form a phthalimido moiety with the nitrogen atom to which they are attached;

10        each R<sub>11</sub> is, independently, substituted or unsubstituted  
C<sub>1</sub>-C<sub>10</sub> alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy,  
t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-  
ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-  
butyryl, phenyl or aryl;

15             $R_5$  is T-L,

T is a bond or a linking moiety;

L is a chemical functional group, a conjugate group or a solid support material;

each  $R_1$  and  $R_2$  is, independently, H, a nitrogen  
20 protecting group, substituted or unsubstituted  $C_1$ - $C_{10}$  alkyl,  
substituted or unsubstituted  $C_2$ - $C_{10}$  alkenyl, substituted or  
unsubstituted  $C_2$ - $C_{10}$  alkynyl, wherein said substitution is  $OR_3$ ,  
 $SR_3$ ,  $NH_3^+$ ,  $N(R_3)(R_4)$ , guanidino or acyl where said acyl is an  
acid amide or an ester;

25           or R<sub>1</sub> and R<sub>2</sub>, together, are a nitrogen protecting group  
or are joined in a ring structure that optionally includes an  
additional heteroatom selected from N and O;

or  $R_1$ , T and L, together, are a chemical functional group;

30           each R<sub>3</sub> and R<sub>4</sub> is, independently, H, C<sub>1</sub>-C<sub>10</sub> alkyl, a  
nitrogen protecting group, or R<sub>3</sub> and R<sub>4</sub>, together, are a  
nitrogen protecting group;

or R<sub>3</sub> and R<sub>4</sub> are joined in a ring structure that optionally includes an additional heteroatom selected from N and O;



$$Z_4 \text{ is } OX, SX, \text{ or } N(X)_2;$$

each X is, independently, H, C<sub>1</sub>-C<sub>8</sub> alkyl, C<sub>1</sub>-C<sub>8</sub> haloalkyl, C(=NH)N(H)R<sub>5</sub>, C(=O)N(H)R<sub>5</sub> or OC(=O)N(H)R<sub>5</sub>;

R<sub>5</sub> is H or C<sub>1</sub>-C<sub>8</sub> alkyl;

5           Z<sub>1</sub>, Z<sub>2</sub> and Z<sub>3</sub> comprise a ring system having from about 4  
to about 7 carbon atoms or having from about 3 to about 6  
carbon atoms and 1 or 2 hetero atoms wherein said hetero atoms  
are selected from oxygen, nitrogen and sulfur and wherein said  
ring system is aliphatic, unsaturated aliphatic, aromatic, or  
10 saturated or unsaturated heterocyclic;

Z<sub>5</sub> is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, N(R<sub>1</sub>)(R<sub>2</sub>) OR<sub>1</sub>, halo, SR<sub>1</sub> or CN;

15        each  $q_1$  is, independently, an integer from 1 to 10;  
           each  $q_2$  is, independently, 0 or 1;  
            $q_3$  is 0 or an integer from 1 to 10;  
            $q_4$  is an integer from 1 to 10;  
            $q_5$  is from 0, 1 or 2; and

20 provided that when  $q_3$  is 0,  $q_4$  is greater than 1.

Representative substituent groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled "Capped 2'-Oxyethoxy Oligonucleotides," hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled "RNA Targeted 2'-Modified Oligonucleotides that are Conformationally Preorganized," hereby incorporated by reference in its entirety.

Particularly preferred substituent groups include  $O[(CH_2)_nO]_mCH_3$ ,  $O(CH_2)_nOCH_3$ ,  $O(CH_2)_nNH_2$ ,  $O(CH_2)_nCH_3$ ,  $O(CH_2)_nONH_2$ ,  $O(CH_2)_nON[(CH_2)_nCH_3]_2$  (where n and m are from 1 to about 10),  $C_1$  to  $C_{10}$  lower alkyl, substituted lower alkyl, alkaryl, aralkyl,

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on nucleosides and oligomers, particularly the 3' position of the sugar on the 3' terminal nucleoside or at a 3'-position of a nucleoside that has a linkage from the 2'-position such as a 2'-5' linked oligomer and at the 5'-position at a 5'-terminus. Oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/468,037, filed on June 5, 1995, also herein incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on nucleosides and oligomers, particularly the 3' position of the sugar on the 3' terminal nucleoside or at a 3'-position of a nucleoside that has a linkage from the 2'-position such as a 2'-5' linked oligomer and at the 5'-position at a 5'-terminus. Oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/468,037, filed on June 5, 1995, also herein incorporated by reference.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by  
5 reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent Application 09/XXX,XXX, entitled "2'-O-Acetamido Modified Monomers and Oligomers", filed August 19, 1999, also identified by attorney docket number ISIS-4071, hereby incorporated by reference in its entirety.

Representative dimethylaminoethoxyethyl substituent groups are disclosed in International Patent Application PCT/US99/XXXXX, entitled "2'-O-Dimethylaminoethoxyethyl-  
15 Modified Oligonucleotides", filed August 6, 1999, also identified by attorney docket number ISIS-4045, hereby incorporated by reference in its entirety.

The use of mixed modifications in the external regions to impart nuclease resistance to oligomeric compounds is also within the scope of the present invention. For example an oligomeric compound of the invention can have enhanced nuclease resistance resulting from one or more modified internucleoside linkages at the 5' end and one or more substituent groups at the 3' end. Another type of a mixed modification includes having a modified internucleoside linkage and a substituent group at the same end of a selected oligomeric compound. Other examples include substituent groups or modified linkages used in conjunction with a non-standard linkage such as a 2', 5'-internucleoside linkage.

30 Oligomeric compounds of the present invention comprise two external regions each having at least one nucleoside and one internucleoside linkage flanking a chiral Rp phosphorothioate internal region. There can be a plurality of up to about 50 linked nucleosides in each external region. A more

preferred range is from 1 to about 6 with 1 to about 3 being more preferred.

The chiral Rp 2'-deoxyphosphorothioate internal region can comprise a plurality of nucleosides with modern techniques 5 allowing routine synthesis of oligomeric compounds having well over 100 nucleosides. A preferred range is from about 3 to about 48 with from about 5 to about 28 being more preferred. An even more preferred range is from about 12 to about 23 nucleosides.

10 Oligomeric compounds according to the present invention  
that are hybridizable to a target nucleic acid preferably  
comprise from about 5 to about 50 nucleosides. It is more  
preferred that such compounds comprise from about 8 to about  
30 nucleosides, with 15 to 25 nucleosides being particularly  
15 preferred.

Cleavage of oligomeric compounds by nucleolytic enzymes requires the formation of an enzyme-substrate complex, or in particular, a nuclease-oligomer complex. The nuclease enzymes will generally require specific binding sites located on the oligomers for appropriate attachment. If the oligomer binding sites are removed or blocked, such that nucleases are unable to attach, the oligomers will be nuclease resistant. In the case of restriction endonucleases that cleave sequence-specific palindromic double-stranded DNA, certain binding sites such as the ring nitrogen in the 3- and 7-positions of heterocyclic base moieties have been identified as required binding sites. Removal of one or more of these sites or sterically blocking approach of the nuclease to these particular positions within the oligomer has provided various levels of resistance to specific nucleases.

In the context of this invention, "hybridization" shall mean hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleosides. For example, adenine and thymine are  
35 complementary nucleobases which pair through the formation of

hydrogen bonds. "Complementary," as used herein, also refers to sequence complementarity between two nucleotides. For example, if a nucleotide at a certain position of an oligomeric compound is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligomeric compound and the DNA or RNA are considered to be complementary to each other at that position. The oligomeric compound and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleosides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligomeric compound and the DNA or RNA target. It is understood that an oligomeric compound need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligomeric compound is specifically hybridizable when binding of the oligomeric compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, i.e. under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

It is known from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, 1970, 47, 1504) and analysis of crystals of double-stranded nucleic acids that DNA takes a "B" form structure and RNA takes the more rigid "A" form structure. The difference between the sugar puckering (C2' endo for "B" form DNA and C3' endo for "A" form RNA) of the nucleosides of DNA and RNA is

the major conformational difference between double-stranded nucleic acids.

The primary contributor to the conformation of the pentofuranosyl moiety is the nature of the substituent at the 2'-position. Thus, the population of the C3'-endo form increases with respect to the C2'-endo form as the electronegativity of the 2'-substituent increases. For example, among 2'-deoxy-2'-haloadenosines, the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). Those of adenosine (2'-OH) and deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-adenosine) is further correlated to the stabilization of the stacked conformation. Research indicates that dinucleoside phosphates have a stacked conformation with a geometry similar to that of A-A but with a greater extent of base-base overlapping than A-A. It is assumed that the highly polar nature of the C2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an "A" structure.

Data from UV hypochromicity, circular dichroism, and <sup>1</sup>H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an "A" form duplex than a "B" form duplex.

Thus, a 2'-substituent on the 3'-nucleotidyl unit of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent.

Studies with a 2'-OMe modification of 2'-deoxy guanosine, cytidine, and uridine dinucleoside phosphates exhibit enhanced stacking effects with respect to the corresponding unmethylated species (2'-OH). In this case, it is believed that the hydrophobic attractive forces of the methyl group tend to overcome the destabilizing effects of its steric bulk.

Melting temperatures (complementary binding) are increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

A heterocyclic base moiety (often referred to in the art simply as a "base" or a "nucleobase") amenable to the present invention includes both naturally and non-naturally occurring nucleobases. The heterocyclic base moiety further may be protected wherein one or more functionalities of the base bears a protecting group. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine and guanine, and the pyrimidine bases thymine, cytosine and uracil. Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine

and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993.

10 Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-  
15 propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C (*Id.*, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-methoxyethyl sugar modifications.

20 Representative United States patents that teach the preparation of modified nucleobases include, but are not limited to, U.S. Patents 3,687,808; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540;  
25 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/762,488, filed on December 10, 1996, also herein incorporated by reference.

30 The attachment of conjugate groups to oligomers is well documented in the prior art. The oligomeric compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. In a preferred embodiment conjugate groups are  
35 attached at one of the 5' or 3'-terminal ends of oligomers of



the invention. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that

5 enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this

10 invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake,

15 distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992, United States Patent No. 5,578,718, issued July 1, 1997, and United States Patent No. 5,218,105. Each of the foregoing is

20 commonly assigned with this application. The entire disclosure of each is incorporated herein by reference.

Preferred conjugate groups amenable to the present invention include lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86,

25 6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992,

30 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 111; Kabanov et al., *FEBS Lett.*, 1990, 259, 327; Svinarchuk et al., *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium-1,2-di-O-hexadecyl-rac-

glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969), adamantane  
5 acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923).

Other groups that can be attached to oligomeric compounds of the invention to modify antisense properties include RNA cleaving complexes, pyrenes, metal chelators, porphyrins, alkylators, hybrid intercalator/ligands and photo-crosslinking agents. RNA cleavers include o-phenanthroline/Cu complexes and  $\text{Ru}(\text{bipyridine})_3^{2+}$  complexes. The  $\text{Ru}(\text{bpy})_3^{2+}$  complexes interact with nucleic acids and cleave nucleic acids photochemically. Metal chelators include EDTA, DTPA, and o-phenanthroline. Alkylators include compounds such as iodoacetamide. Porphyrins include porphine, its substituted forms, and metal complexes. Pyrenes include pyrene and other pyrene-based carboxylic acids that could be conjugated using the similar protocols.

Hybrid intercalator/ligands include the  
photonuclease/intercalator ligand 6-[[[9-[[6-(4-nitro-  
25 benzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-  
pentafluorophenyl ester. This compound has two noteworthy  
features: an acridine moiety that is an intercalator and a p-  
nitro benzamido group that is a photonuclease.

Photo-crosslinking agents include aryl azides such as, 30 for example, N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) and N-succinimidyl-6(-4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH). Aryl azides conjugated to oligonucleotides effect crosslinking with nucleic acids and proteins upon irradiation.

[illegible]

They also crosslink with carrier proteins (such as KLH or BSA), raising antibody against the oligonucleotides.

Vitamins can also be attached to oligomeric compounds of the invention to improve properties such as absorption and distribution. Vitamins according to the invention generally can be classified as water soluble or lipid soluble. Water soluble vitamins include thiamine, riboflavin, nicotinic acid or niacin, the vitamin B<sub>6</sub> pyridoxal group, pantothenic acid, biotin, folic acid, the B<sub>12</sub> cobamide coenzymes, inositol, choline and ascorbic acid. Lipid soluble vitamins include the vitamin A family, vitamin D, the vitamin E tocopherol family and vitamin K (and phytols). The vitamin A family, including retinoic acid and retinol, are absorbed and transported to target tissues through their interaction with specific proteins such as cytosol retinol-binding protein type II (CRBP-II), retinol-binding protein (RBP), and cellular retinol-binding protein (CRBP). These proteins, which have been found in various parts of the human body, have molecular weights of approximately 15 kD. They have specific interactions with compounds of vitamin-A family, especially, retinoic acid and retinol.

As used herein, "polyamine" refers to a moiety containing a plurality of amine or substituted amine functionalities. Polyamines according to the present invention have at least two amine functionalities.

"Polypeptide" refers to a polymer comprising a plurality of amino acids linked by peptide linkages, and includes dipeptides and tripeptides. The amino acids may be naturally-occurring or non-naturally-occurring amino acids. Polypeptides according to the present invention comprise at least two amino acids.

The gapmers of the present invention can include appropriate activated phosphorus groups such as activated phosphate groups and activated phosphite groups. As used herein, the terms activated phosphate and activated phosphite

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groups refer to activated monomers or oligomers that are reactive with a hydroxyl group of another monomeric or oligomeric compound to form a phosphorus-containing internucleotide linkage. Such activated phosphorus groups contain activated phosphorus atoms in  $P^{III}$  or  $P^V$  valency states. Such activated phosphorus atoms are known in the art and include, but are not limited to, phosphoramidite, H-phosphonate and phosphate triesters. A preferred synthetic solid phase synthesis utilizes phosphoramidites as activated phosphates. The phosphoramidites utilize  $P^{III}$  chemistry. The intermediate phosphite compounds are subsequently oxidized to the  $P^V$  state using known methods to yield, in a preferred embodiment, phosphodiester or phosphorothioate internucleotide linkages. Additional activated phosphates and phosphites are disclosed in Tetrahedron Report Number 309 (Beaucage and Iyer, *Tetrahedron*, **1992**, 48, 2223-2311).

Functional groups including substituent groups discussed above which may be located on heterocyclic base and sugar moieties are routinely blocked with protecting (blocking groups) during synthesis and subsequently deblocked. In general, a blocking group renders a chemical functionality of a molecule inert to specific reaction conditions and can later be removed from such functionality in a molecule without substantially damaging the remainder of the molecule. See, Green and Wuts, *Protective Groups in Organic Synthesis*, 2d edition, John Wiley & Sons, New York, 1991. For example, amino groups can be blocked with nitrogen protecting groups such as phthalimido, 9-fluorenylmethoxycarbonyl (Fmoc), triphenylmethylsulfenyl, t-BOC or benzyl groups. Carboxyl groups can be protected as acetyl groups. Representative hydroxyl protecting groups are described by Beaucage et al., *Tetrahedron* **1992**, 48, 2223. Preferred hydroxyl protecting groups are acid-labile groups, such as the trityl, monomethoxytrityl, dimethoxytrityl, trimethoxytrityl, 9-phenylxanthin-9-yl (Pixyl) and 9-(p-methoxyphenyl)xanthin-9-yl

(MOX). Chemical functional groups can also be "blocked" by including them in a precursor form. Thus an azido group can be considered a "blocked" form of an amine as the azido group is easily converted to the amine. Further representative  
 5 protecting groups utilized in oligonucleotide synthesis are discussed in Agrawal *et al.*, *Protocols for Oligonucleotide Conjugates*, Eds., Humana Press, New Jersey, 1994, Vol. 26, pp. 1-72.

The term "nucleoside" as used in connection with this  
 10 invention refers to a monomeric unit made up of a heterocyclic base moiety joined to a sugar moiety or sugar mimetic through a glycosyl linkage. The term "nucleotide" refers to a nucleoside having a phosphate group on its 3' or 5' sugar hydroxyl group.

15 In the context of this invention, the terms "oligomer" and "oligomeric compound" refer to a plurality of naturally-occurring or non-naturally-occurring nucleosides joined together in a specific sequence. The terms "oligomer" and "oligomeric compound" include oligonucleotides,  
 20 oligonucleotide analogs, oligonucleosides and chimeric oligomeric compounds where there are more than one type of internucleoside linkages dividing the oligomeric compound into regions. Whereas the term "oligonucleotide" has a well defined meaning in the art, the term "oligomeric compound" or  
 25 "oligomer" is intended to be broader, inclusive of oligomers having all manner of modifications known in the art. Gapped or chimeric compounds are disclosed in for example, U.S. Patent No. 5,623,065, issued April 22, 1997, the contents of which are incorporated herein by reference.

30 As used herein, the term "oligonucleoside" includes oligomers or polymers containing two or more nucleoside subunits having a non-phosphodiester linking moiety. Oligonucleosides according to the invention have a ribofuranose moiety attached to a nucleobase through a  
 35 glycosyl bond.

Gapmer technology has been developed to incorporate modifications at the ends ("wings") of oligomeric compounds, leaving a phosphorothioate gap in the middle for RNase H activation (Cook, P.D., *Anti-Cancer Drug Des.*, **1991**, 6, 585-607; Monia et al., *J. Biol. Chem.*, **1993**, 268, 14514-14522). In a recent report, the activities of a series of uniformly 2'-O modified 20 mer RNase H-independent oligonucleotides that were antisense to the 5'-cap region of human ICAM-1 transcript in HUVEC cells, were compared to the parent 2'-deoxy phosphorothioate oligonucleotide (Baker et al., *J. Bio. Chem.*, **1997**, 272, 11994-12000). The 2'-MOE/P=O oligomer demonstrated the greatest activity with an IC<sub>50</sub> of 2.1 nM (T<sub>m</sub> = 87.1°C), while the parent P=S oligonucleotide analog had an IC<sub>50</sub> of 6.5 nM (T<sub>m</sub> = 79.2°C). Correlation of activity with binding affinity is not always observed as the 2'-F/P=S (T<sub>m</sub> = 87.9°C) was less active than the 2'-MOE/P=S (T<sub>m</sub> = 79.2°C) by four fold. The RNase H competent 2'-deoxy P=S parent oligonucleotide exhibited an IC<sub>50</sub> = 41 nM.

Gapped oligomeric compounds of the present invention are prepared using solution phase and solid phase techniques known in the art. It may be advantageous to prepare a portion of the gapped oligomer using solution phase methodologies employing enzymatic or chemical methods and then use this piece thus synthesized in solid phase methodologies to form the desired final gapped oligomeric compound.

In one aspect of the present invention oligomeric compounds are prepared using known solid phase methodologies. A first modified or unmodified nucleoside is attached to a solid support preferably via a linkage to the 3'-position. The nucleoside could alternatively be attached to a solid support through the 2'-position as when preparing positionally modified internucleoside linkages. Alternatively the solid support with the desired nucleoside is purchased from a commercial source. This nucleoside will ultimately become the nucleoside at the 3'-end of the final oligomeric compound in a

standard synthesis. The solid support with the nucleoside attached is placed in a reaction vessel such as a glass reactor. One of the hydroxyl groups (preferably the 5'-hydroxyl group) is deprotected and treated with a second  
5 nucleoside having a group reactive with the hydroxyl group (preferably an activated phosphate group) in the presence of an activator such as DBU. The linkage thus formed is optionally oxidized or sulferized.

The cycle is repeated to add additional nucleosides  
10 until the 3'-external region is completed. In some embodiments only one nucleoside comprises each external region. The last activated monomer added to the 3'-external region will contribute the internucleoside linkage joining the 3'-external region to the chiral Rp deoxyphosphorothioate  
15 region (internal region) and will also contribute the 3'-nucleoside for the internal region.

The addition of activated monomers is continued to synthesize the remainder of the internal (uniform chiral Rp deoxyphosphorothioate) region by iterative addition of  
20 nucleosides prepared having chiral auxiliary protecting groups as part of their activated phosphorus groups. After each addition the chiral internucleoside linkage is sulfurized using for example Beaucage reagent. Compounds 3, 14 and 20 are examples of an activated monomers that are incorporated  
25 into oligomers to give chiral Rp deoxyphosphorothioate internucleoside linkages after deprotection.

The synthesis is continued for the 5'-external region by adding further activated monomers in the same manner as for the 3'-external region. The final gapmer is deblocked and  
30 cleaved from the solid support. The deblocking and cleavage steps can be performed concurrently or separately. Traditionally the deblocking and cleavage is performed concurrently by treatment with base such as aqueous  $\text{NH}_4\text{OH}$ .

Preferred internucleoside linkages that are prepared as  
35 illustrated above include:

- phosphorodithioate (-O-P(S)(S)-O-);  
 chiral Sp phosphorothioate (-O-P(S)(O)-O-);  
 phosphoramidate (-O-P(O)(NJ)-O-);  
 alkylphosphonate (-O-P(J)(O)-O-); and  
 5 methylene phosphonate (-CH<sub>2</sub>-P(O)(O)-O-).

To incorporate certain internucleoside linkages at one or both of the 3'- or 5'-ends of gapmers of the invention one or more dimers must be prepared and incorporated. One such internucleoside linkage is MMI (methylene(methylimino)). A  
 10 gapmer of the invention having one MMI internucleoside linkage at each of the 3'- and 5'-ends is prepared by first attaching an MMI dimer to a solid support. Next the internal region is prepared following the procedure illustrated above. The addition of an MMI dimer to the 5'-end requires that the dimer  
 15 be modified to have an activated phosphorus group incorporating a chiral auxiliary protecting group attached to the 2'-position of the 3'-terminal nucleoside of the dimer if it is desired to have that position part of the chiral Rp internal region. The chiral auxiliary group will give, upon  
 20 incorporation of the dimer, a chiral Rp phosphorothioate internucleoside linkage. The dimer is modified to incorporate the activated phosphorus group incorporating a chiral auxiliary protecting group as per the procedures of Examples 4, 16 and 23. The final gapped oligomeric compound prepared  
 25 following this approach will have only nuclease resistant MMI internucleoside linkages separating the terminal 3', and the 5'-nucleosides from the chiral Rp phosphorothioate internal region.

A list of preferred internucleoside linkages that are  
 30 more amenable to incorporation into the external regions of oligomeric compounds of the invention following the dimer strategy discussed above for MMI internucleoside linkages include:

- carbamate (-O-C(O)-NH- and -NH-C(O)-O-)  
 35 sulfonamide (-O-SO<sub>2</sub>-NH-);



- N,N'-dimethylhydrazine ( $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-$ );  
thioformacetal ( $-\text{S}-\text{CH}_2-\text{O}-$ );  
formacetal ( $-\text{O}-\text{CH}_2-\text{O}-$ );  
hydroxylamine ( $-\text{CH}_2-\text{N}(\text{J})-\text{O}-$ );  
5 amide ( $-\text{CH}_2-\text{N}(\text{J})-\text{C}(\text{O})-$ ), ( $-\text{CH}_2-\text{C}(\text{O})-\text{N}(\text{J})-$ ); and  
oxime ( $-\text{CH}_2-\text{O}-\text{N}=\text{CH}-$ ) where J is H or alkyl.

In one aspect of the present invention, gapped  
oligimeric compounds are prepared using enzymatic processes or  
a combination of enzymatic and chemical processes. In 1985,  
10 it was reported that a variety of DNA polymerases are capable  
of accepting 2'-deoxynucleoside 5'-O-(1-thiotriphosphates)  
(dNTP- $\alpha$ S) of the Sp-configuration as substrates to produce  
oligonucleotides containing phosphorothioate linkages having  
the Rp-configuration (Eckstein et al., *Annu. Rev. Biochem.*,  
15 **1985**, 54, 367). This property of DNA polymerases has been  
utilized extensively to produce antisense oligonucleotides of  
desired length in high purity (Tang et al., *Nucleosides &*  
*Nucleotides*, **1995**, 14, 985,). The foregoing synthesis was  
based on a primer extension with a polymerase and dNTP- $\alpha$ S as a  
20 substrate. Recently, an oligonucleotide library with  
phosphorothioate backbone was synthesized by PCR amplification  
of a template using commercial Taq polymerase and dNTPS (King  
*ibid*).

As an alternative, RNA ligase have been used with 3'-O-  
25 protected nucleotides as a substrate for the synthesis of  
oligonucleotides (Hyman *ibid*). RNA ligases are ubiquitous and  
promote the joining of single-stranded oligonucleotides in the  
absence of a complementary template strand. For example,  
phage T4 RNA ligase has been reported to connect a 5'-  
30 phosphate to a 3'-hydroxyl group in vitro, which makes this  
enzyme valuable for ligating oligonucleotides derived  
synthetically or enzymatically. RNA ligase can use ATP or  
dATP as its energy source for the ligation reactions. A wide  
variety of naturally occurring and non-naturally occurring

e.g. synthetic nucleotide analogues, are accepted as substrates. This has enabled enzymatic synthesis of oligomeric compounds having nucleosides with modified sugar and base moieties (Brennan et al., *Nucleic Acids Res.*, **1985**, 5 13, 8665).

In addition to the reported ligation of an oligonucleotide to another oligonucleotide, RNA ligase can also add a single nucleotide residue to the 3'-end of a preformed oligonucleotide (Brennan et al. *Methods Enzymol.*, **1983**, 100, 10 38). A common use for this reaction is termed "3'-end labeling." In this process, an oligonucleotide is radio-labeled by the RNA ligase-catalyzed addition of a radioactive 3',5'-bisphosphate nucleotide. The result is a radioactively labeled oligomer that is one nucleotide longer that carries a 15 3'-phosphate. This reaction is very clean and there is no further extension of the oligonucleotide because the 3'-phosphate group acts as a protecting group. Addition of further nucleotides is possible by selectively removing the 3'-phosphate group with alkaline phosphatase. Like RNA 20 ligase, alkaline phosphatase is ubiquitous and commonly isolated from calf intestine. A similar use of alkaline phosphatase has also been reported for the synthesis of oligonucleotides (Hyman *ibid*).

In one aspect of the present invention a gapped 25 oligomeric compound is prepared using methods and techniques illustrated in the prior art. To prepare a gapped oligomeric compound a first external region is prepared having one or more nucleoside residues either by standard chemical or enzymatic techniques. This first external region is connected 30 or ligated by RNA ligase to an internal region. The internal region is easily prepared using enzymatic methods. Subsequently, the larger fragments can be either purified if necessary or further ligated to another monomer or oligomer to furnish a much larger oligomeric molecule. This process can 35 be repeated until a desired gapped oligomeric compound is

prepared having a desired length and sequence. Therefore, one can take advantage of the all-Rp isomer made via enzymatic method and enhance its stability in vivo by end capping with a modified residue synthesized separately.

5           Enzymatic synthesis of an all-Rp phosphorothioate oligonucleotide core may have several advantages over the traditional automated phosphoramidite chemistry. For example, the coupling reaction can be performed at a lower concentration of enzymes compared to the hazardous reagents,  
10 such as dichloromethane and 1-H tetrazole required for the automated synthesis. Also, enzymatic reactions can be carried out at much higher oligomer concentration compared to the solid-phase approach which is currently performed at a 150 mmol scale. This can result in more product yield per  
15 synthesis cycle and will eventually help reduce the labor cost. From an environmental point of view, the enzymatic route is superior when compared to the synthetic route because it does not employ any organic solvents and can avoid waste disposal of complex non-biodegradable reagents. The key raw  
20 materials for solid-support synthesis are amidites which are difficult to synthesize and costly. The necessary nucleotides required for enzymatic methods can be prepared through a more direct and economical enzymatic method.

In one aspect of the present invention gapped oligomeric  
25 compounds of formulas 48-51 (Figures 14 and 15) are prepared enzymatically. The internal all-Rp region is prepared following one of the standard enzymatic methods followed by ligation of the external regions.

For example, in order to synthesize a gapped oligomeric  
30 compound having for example formula 49 (Figure 15) that is capped on both the 3'- and the 5'-ends with nucleosides having modified internucleoside linkages "L", such as MMI, the following protocol can be used. An appropriately protected MMI dimer 28 (Figure 8) with L being methylene(methylimino)  
35 can be synthesized following the literature protocol (Bhat et

al. *J. Org. Chem.*, **1996**, *61*, 8186) that will become the 5'-end of the molecule. In the first step of the enzymatic synthesis the MMI dimer having containing a 3'-hydroxyl group is reacted with an activated pyrophosphate Formula 31 (Figure 8) to  
5 furnish a compound having Formula 39 (Figure 11) with a single Rp phosphorothioate internucleoside linkage. This coupling reaction is performed in the presence of RNA ligase and ATP as a source of energy.

The compound having Formula 39 is then treated with  
10 alkaline phosphatase to remove the 3'-protecting group (Pg<sub>2</sub>, a phosphate group). Cleavage of 3'-phosphate group furnishes a compound having formula 41 (Figure 11) containing a 3'-hydroxyl group. The RNA ligase mediated coupling reaction is repeated with a further nucleoside pyrophosphate to give a  
15 compound having Formula 43 (Figure 11). The deprotection and subsequent coupling reactions are then repeated a desired number of times until a desired number and sequence of Rp phosphorothioate linkages are assembled. The deprotection step is repeated to give a compound having Formula 45 (Figure  
20 13) having a free 3' hydroxyl group. The free 3'-hydroxyl group is then ligated with an MMI modified pyrophosphate in the presence of RNA ligase, as described above, to provide the protected gapped oligomeric compound having formula 47 (Figure 13) with a single MMI linkage on each of the 3'- and 5'-  
25 termini. Purification by for example reverse phase chromatography followed by deprotection of the Pg<sub>1</sub> groups will give the final gapped oligomeric compound having Formula 49 (Figure 15).

Phosphorothioate oligonucleotides having chirally pure  
30 intersugar linkages may be analyzed in a number of ways. For example, configuration analysis of resulting sequence specific phosphorothioate oligonucleotides having substantially chirally pure all-Sp or all-Rp intersugar linkages may be determined by the use of [<sup>31</sup>P] NMR chemical shifts. Such  
35 chemical shifts have been used to identify the Rp epimer of a

phosphorothioate di-nucleotide (Ludwig et al., *J. Org. Chem.*, 1989, 54, 631-635).

The fidelity of sequences of phosphorothioate oligonucleotides of the invention can be determined using the sensitivities of heteroduplexes to S1 nuclease. The sequence of the phosphorothioate oligonucleotides can be further substantiated by labeling the 3'hydroxyls of phosphorothioate oligonucleotides with [ $\alpha$ - $^{32}$ P]cordycepin triphosphate, i.e. 3'-deoxyadenosine-5'-triphosphate. The resultant oligomeric compounds may be subjected to enzymatic degradation.

The relative ability of oligomeric compounds having regions of chirally pure intersugar linkages, as compared to the identical racemic sequences, to bind to complementary nucleic acid strands is compared by determining the melting temperature of a hybridization complex of each oligomer with its complementary strand. The melting temperature ( $T_m$ ), a characteristic physical property of double helixes, denotes the temperature in degrees centigrade at which 50% helical versus coiled (unhybridized) forms are present.  $T_m$  is measured by using the UV spectrum to determine the formation and breakdown (melting) of hybridization. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently a reduction in UV absorption indicates a higher  $T_m$ . The higher the  $T_m$ , the greater the strength of the binding of the strands. Non Watson-Crick base pairing has a strong destabilizing effect on the  $T_m$ . Consequently, as close to optimal fidelity of base pairing as possible is desired to have optimal binding of an oligomer to its targeted RNA.

Oligomeric compounds of the invention are also evaluated as to their resistance to degradation by a variety of exonucleases and endonucleases. Oligomers of the invention are treated with nucleases and then analyzed, as for instance, by polyacrylamide gel electrophoresis (PAGE) followed by staining with a suitable stain such as Stains All<sup>TM</sup> (Sigma

Chem. Co., St. Louis, MO). Degradation products are quantitated using laser densitometry.

The sensitivity of heteroduplexes formed from oligomeric compounds of the invention with target nucleic acid to catalytic activity of RNase H is also easily assessed. An oligomeric compound is incubated with a radiolabeled target mRNA (synthesized as for instance via T7 RNA polymerase) at various temperatures for hybridization. Heteroduplexes can then be incubated at 37°C with RNase H from *E. coli* according to the procedure of Minshull, J. and Hunt, T., *Nuc. Acid Res.*, **1986**, 6433-6451. Products are then assessed for RNase H activity by Northern Blot analysis wherein products are electrophoresed on a 1.2% agarose/ formaldehyde gel and transferred to nitrocellulose. Filters are then probed using a random primer [<sup>32</sup>P]-labeled cDNA complementary to target mRNA and quantitated by autoradiography. The effect of modifications in the external regions as compared to unmodified gapmers on the relative ability of the resultant heteroduplex to act as a substrate for RNase H is then calculated for various modifications.

Comparisons of the susceptibility of heteroduplexes to the catalytic action of *E. coli* RNase H and mammalian RNase H are performed. Heteroduplexes are incubated in rabbit reticulocyte lysates under conditions of translation and assayed via Northern blot analysis for catalytic cleavage of mRNA by endogenous RNase H.

As used herein the term "chiral auxiliary" is meant to include groups that function to provide chirality to internucleoside phosphorus linkages during the course of the synthesis of oligomeric phosphorothioates. Chiral auxiliaries will give either Sp or Rp chirality for the respective internucleoside linkage in the final oligomeric compound. Accordingly, chiral auxiliaries are allowed to remain on the growing chain, and are removed at the end of the iterative synthetic regime. Removal of chiral auxiliaries can be

conveniently accomplished in a single treatment after the completion of the iterative synthesis.

As used herein, the term "alkyl" includes, but is not limited to, straight chain, branched chain and alicyclic hydrocarbon groups. Alkyl groups of the present invention may be substituted. Representative alkyl substituents are disclosed in United States Patent No. 5,212,295, at column 12, lines 41-50, hereby incorporated by reference in its entirety. Substituent groups include, but are not limited to, alkyl, alkenyl, alkynyl, aryl, hydroxyl, alkoxy, alcohol, benzyl, phenyl, nitro, thiol, thioalkoxy, thioalkyl, trifluoromethyl, halo, nitrile, trifluoromethoxy and azido. As used herein, the term "lower alkyl" is intended to mean an alkyl group having 10 or fewer carbons.

Alkenyl groups according to the invention are to straight chain, branch chain, and cyclic hydrocarbon groups containing at least one carbon-carbon double bond, and alkynyl groups are to straight chain, branch chain, and cyclic hydrocarbon groups containing at least one carbon-carbon triply bond. Alkenyl and alkynyl groups of the present invention can be substituted.

Aryl groups are substituted and unsubstituted aromatic cyclic moieties including but not limited to phenyl, naphthyl, anthracyl, phenanthryl, pyrenyl, and xylyl groups. Alkaryl groups are those in which an aryl moiety links an alkyl moiety to a core structure, and aralkyl groups are those in which an alkyl moiety links an aryl moiety to a core structure.

As used herein, the term "aralkyl" denotes alkyl groups which bear aryl groups, for example, benzyl groups. The term "alkaryl" denotes aryl groups which bear alkyl groups, for example, methylphenyl groups. As used herein, the term "aryl" denotes aromatic cyclic groups including, but not limited to, phenyl, naphthyl, anthracyl, phenanthryl and pyrenyl.

Preferred aryl and aralkyl groups include, but are not limited to, phenyl, benzyl, xylyl, naphthyl, toluyl, pyrenyl,

anthracyl, azulyl, phenethyl, cinnamyl, benzhydryl, and mesityl. Typical substituents for substitution include, but are not limited to, hydroxyl, alkoxy, alcohol, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, or alkyl, aryl, alkenyl, or  
5 alkynyl groups.

As used herein, the term "alkanoyl" has its accustomed meaning as a group of formula  $-C(=O)-alkyl$ . A preferred alkanoyl group is the acetyl group.

For therapeutic or pharmaceutical use, the oligomeric  
10 compounds of the present invention may be taken up in pharmaceutically acceptable carriers such as, for example, solutions, suspensions, tablets, capsules, ointments, elixirs and injectable compositions. The dosage administered depends upon factors such as the nature and severity of the condition,  
15 the stage of the condition, and the condition of the patient. An effective amount of oligomeric compound of the invention may be from about  $10\mu g/kg$  body weight to about  $1000\mu g/kg$  body weight.

The oligomeric compounds of the present invention can be  
20 used in diagnostics, therapeutics and as research reagents and kits. The oligomeric compounds of the present invention can also be used in pharmaceutical compositions by including a suitable pharmaceutically acceptable diluent or carrier. The oligomeric compounds can further be used for treating  
25 organisms having a disease characterized by the undesired production of a protein. For this purpose, the organism is contacted with an oligomer having a sequence that is capable of specifically hybridizing with a strand of nucleic acid encoding the undesirable protein. Treatments of this type can  
30 be practiced on a variety of organisms ranging from unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes DNA-RNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular control is  
35 susceptible to therapeutic and/or prophylactic treatment in



accordance with the invention. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, all plants and all higher animal forms, including warm-blooded animals, can be treated. Further, each cell of multicellular eukaryotes can  
5 be treated, as they include both DNA-RNA transcription and RNA-protein translation as integral parts of their cellular activity. Furthermore, many of the organelles (e.g., mitochondria and chloroplasts) of eukaryotic cells also include transcription and translation mechanisms. Thus, single cells,  
10 cellular populations or organelles can also be included within the definition of organisms that can be treated with therapeutic or diagnostic oligomeric compounds of the invention.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill  
15 of those in the art. In general, for therapeutics, a patient in need of such therapy is administered an oligomer in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01  $\mu$ g to 100 g per kg of body weight depending on the age of the patient and the  
20 severity of the disease state being treated. Further, the treatment may be a single dose or may be a regimen that may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient, and may extend from once daily to  
25 once every 20 years. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disease state. The dosage of the oligomer may either be increased in the event the patient does not respond significantly to current dosage levels, or the  
30 dose may be decreased if an alleviation of the symptoms of the disease state is observed, or if the disease state has been abated.

In some cases it may be more effective to treat a patient with an oligomer of the invention in conjunction with  
35 other traditional therapeutic modalities. For example, a

patient being treated for AIDS may be administered an oligomer in conjunction with AZT, or a patient with atherosclerosis may be treated with an oligomer of the invention following angioplasty to prevent reocclusion of the treated arteries.

5        Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from  
10 measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligomers, and can generally be estimated based on  
15 EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to several years.

Following successful treatment, it may be desirable to  
20 have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligomer is administered in maintenance doses, ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every several years.

25        The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral.  
30 Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops,  
35 suppositories, sprays, liquids and powders. Conventional

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pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or  
5 granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for intrathecal or intraventricular  
10 administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

15 There are a many therapeutic indications and general uses for oligomeric compounds of the present invention. Representative indications and uses include the following:

One therapeutic indication of particular interest is psoriasis. Psoriasis is a common chronic and recurrent  
20 disease characterized by dry, well-circumscribed, silvery, scaling papules and plaques of various sizes. The disease varies in severity from a few lesions to widespread dermatosis with disabling arthritis or exfoliation. The ultimate cause of psoriasis is not known, but the thick scaling that occurs  
25 is probably due to increased epidermal cell proliferation (*The Merck Manual of Diagnosis and Therapy*, 15th Ed., pp. 2283-2285, Berkow et al., eds., Rahway, N.J., 1987). Inhibitors of Protein Kinase C (PKC) have been shown to have both antiproliferative and anti-inflammatory effects *in vitro*.  
30 Some antipsoriasis drugs, such as cyclosporin A and anthralin, have been shown to inhibit PKC, and inhibition of PKC has been suggested as a therapeutic approach to the treatment of psoriasis (Hegemann, L. and G. Mahrle, *Pharmacology of the Skin*, H. Mukhtar, ed., pp. 357-368, CRC Press, Boca Raton, FL,  
35 1992). Antisense compounds targeted to Protein Kinase C (PKC)

proteins are described in U.S. Patents Nos. 5,620,963 to Cook et al. and 5,681,747 to Boggs et al.

Another type of therapeutic indication of interest is inflammatory disorders of the skin. These occur in a variety of forms including, for example, lichen planus, toxic epidermal necrolysis (TEN), erythema multiforme and the like (*The Merck Manual of Diagnosis and Therapy*, 15th Ed., pp. 2286-2292, Berkow et al., eds., Rahway, N.J., 1987). Expression of ICAM-1 has been associated with a variety of inflammatory skin disorders such as allergic contact dermatitis, fixed drug eruption, lichen planus and psoriasis (Ho et al., *J. Am. Acad. Dermatol.*, 1990, 22, 64; Griffiths et al., *Am. J. Pathology*, 1989, 135, 1045; Lisby et al., *Br. J. Dermatol.*, 1989, 120, 479; Shiohara et al., *Arch. Dermatol.*, 1989, 125, 1371; Regezi et al., *Oral Surg. Oral Med. Oral Pathol.*, 1996, 81, 682). Moreover, intraperitoneal administration of a monoclonal antibody to ICAM-1 decreases ovalbumin-induced eosinophil infiltration into skin in mice (Hakugawa et al., *J. Dermatol.*, 1997, 24, 73). Antisense compounds targeted to ICAM-1 are described in U.S. Patents Nos. 5,514,788 and 5,591,623, and co-pending U.S. patent applications Serial Nos. 09/009,490 and 09/062,416, January 20, 1998 and April 17, 1998, respectively, all to Bennett et al.

Other antisense targets for skin inflammatory disorders are VCAM-1 and PECAM-1. Intraperitoneal administration of a monoclonal antibody to VCAM-1 decreases ovalbumin-induced eosinophil infiltration into the skin of mice (Hakugawa et al., *J. Dermatol.*, 1997, 24, 73). Antisense compounds targeted to VCAM-1 are described in U.S. Patents Nos. 5,514,788 and 5,591,623. PECAM-1 proteins are glycoproteins which are expressed on the surfaces of a variety of cell types (for reviews, see Newman, *J. Clin. Invest.*, 1997, 99, 3 and DeLisser et al., *Immunol. Today*, 1994, 15, 490). In addition

to directly participating in cell-cell interactions, PECAM-1 apparently also regulates the activity and/or expression of other molecules involved in cellular interactions (Litwin et al., *J. Cell Biol.*, 1997, 139, 219) and is thus a key mediator of several cell:cell interactions. Antisense compounds targeted to PECAM-1 are described in co-pending U.S. patent application Serial No. 09/044,506, filed March 19, 1998, by Bennett et al.

Another type of therapeutic indication of interest for oligonucleotides encompasses a variety of cancers of the skin. Representative skin cancers include benign tumors (warts, moles and the like) and malignant tumors such as, for example, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Paget's disease, Kaposi's sarcoma and the like (*The Merck Manual of Diagnosis and Therapy*, 15th Ed., pp. 2301-2310, Berkow et al., eds., Rahway, N.J., 1987). A number of molecular targets involved in tumorigenesis, maintenance of the hyperproliferative state and metastasis are targeted to prevent or inhibit skin cancers, or to prevent their spread to other tissues.

The *ras* oncogenes are guanine-binding proteins that have been implicated in cancer by, e.g., the fact that activated *ras* oncogenes have been found in about 30% of human tumors generally; this figure approached 100% in carcinomas of the exocrine pancreas (for a review, see Downward, *Trends in Biol. Sci.*, 1990, 15, 469). Antisense compounds targeted to H-*ras* and K-*ras* are described in U.S. Patent No. 5,582,972 to Lima et al., 5,582,986 to Monia et al. and 5,661,134 to Cook et al., and in published PCT application WO 94/08003.

Protein Kinase C (PKC) proteins have also been implicated in tumorigenesis. Antisense compounds targeted to Protein Kinase C (PKC) proteins are described in U.S. Patents Nos. 5,620,963 to Cook et al. and 5,681,747 to Boggs et al. Also of interest are AP-1 subunits and JNK proteins,

particularly in regard to their roles in tumorigenesis and metastasis. The process of metastasis involves a sequence of events wherein (1) a cancer cell detaches from its extracellular matrices, (2) the detached cancer cell migrates to another portion of an animal's body, often via the circulatory system, and (3) attaches to a distal and inappropriate extracellular matrix, thereby created a focus from which a secondary tumor can arise. Normal cells do not possess the ability to invade or metastasize and/or undergo apoptosis (programmed cell death) if such events occur (Ruoslahti, *Sci. Amer.*, 1996, 275, 72). However, many human tumors have elevated levels of activity of one or more matrix metalloproteinases (MMPs) (Stetler-Stevenson et al., *Annu. Rev. Cell Biol.*, 1993, 9, 541; Bernhard et al., *Proc. Natl. Acad. Sci. (U.S.A.)*, 1994, 91, 4293. The MMPs are a family of enzymes which have the ability to degrade components of the extracellular matrix (Birkedal-Hansen, *Current Op. Biol.*, 1995, 7, 728). In particular, one member of this family, matrix metalloproteinase-9 (MMP-9), is often found to be expressed only in tumors and other diseased tissues (Himelstein et al., *Invasion & Metastasis*, 1994, 14, 246).

Several studies have shown that regulation of the MMP-9 gene may be controlled by the AP-1 transcription factor (Kerr et al., *Science*, 1988, 242, 1242; Kerr et al., *Cell*, 1990, 61, 267; Gum et al., *J. Biol. Chem.*, 1996, 271, 10672; Hua et al., *Cancer Res.*, 1996, 56, 5279). Inhibition of AP-1 function has been shown to attenuate MMP-9 expression (U.S. patent application Serial No. 08/837,201). AP-1 is a heterodimeric protein having two subunits, the gene products of *fos* and *jun*. Antisense compounds targeted to *c-fos* and *c-jun* are described in co-pending U.S. patent application Serial No. 08/837,201, filed March 14, 1997, by Dean et al.

Furthermore, AP-1 is itself activated in certain circumstances by phosphorylation of the Jun subunit at an

amino-terminal position by Jun N-terminal kinases (JNKs). Thus, inhibition of one or more JNKs is expected to result in decreased AP-1 activity and, consequentially, reduced MMP expression. Antisense compounds targeted to JNKs are

5 described in co-pending U.S. patent application Serial No. 08/910,629, filed August 13, 1997, by Dean *et al.*

Infectious diseases of the skin are caused by viral, bacterial or fungal agents. In the case of Lyme disease, the tick borne causative agent thereof, the spirochete *Borrelia burgdorferi*, up-regulates the expression of ICAM-1, VCAM-1 and ELAM-1 on endothelial cells in vitro (Boggemeyer et al., *Cell Adhes. Comm.*, 1994, 2, 145). Furthermore, it has been proposed that the mediation of the disease by the anti-inflammatory agent prednisolone is due in part to mediation of this up-regulation of adhesion molecules (Hurtenbach et al., *Int. J. Immunopharmac.*, 1996, 18, 281). Thus, potential targets for therapeutic mediation (or prevention) of Lyme disease include ICAM-1, VCAM-1 and ELAM-1 (*supra*).

Other infectious disease of the skin which are tractable  
20 to treatment using the compositions and methods of the  
invention include disorders resulting from infection by  
bacterial, viral or fungal agents (*The Merck Manual of  
Diagnosis and Therapy*, 15th Ed., pp. 2263-2277, Berkow et al.,  
eds., Rahway, N.J., 1987). With regards to infections of the  
25 skin caused by fungal agents, U.S. Patent 5,691,461 provides  
antisense compounds for inhibiting the growth of *Candida  
albicans*.

With regards to infections of the skin caused by viral agents, U.S. Patent 5,166,195, 5,523,389 and 5,591,600 provide oligonucleotide inhibitors of Human Immunodeficiency Virus (HIV). U.S. Patent 5,004,810 provides oligomers capable of hybridizing to herpes simplex virus Vmw65 mRNA and inhibiting its replication. U.S. Patent 5,194,428 and 5,580,767 provide antisense compounds having antiviral activity against

influenzavirus. U.S. Patent 4,806,463 provides antisense compounds and methods using them to inhibit HTLV-III replication. U.S. Patents 4,689,320, 5,442,049, 5,591,720 and 5,607,923 are directed to antisense compounds as antiviral agents specific to cytomegalovirus (CMV). U.S. Patent 5,242,906 provides antisense compounds useful in the treatment of latent Epstein-Barr virus (EBV) infections. U.S. Patents 5,248,670, 5,514,577 and 5,658,891 provide antisense compounds useful in the treatment of herpesvirus infections. U.S. Patents 5,457,189 and 5,681,944 provide antisense compounds useful in the treatment of papillomavirus infections. The antisense compounds disclosed in these patents, which are herein incorporated by reference, may be used with the compositions of the invention to effect prophylactic, palliative or therapeutic relief from diseases caused or exacerbated by the indicated pathogenic agents.

Antisense oligomeric compounds employed in the compositions of the present invention may also be used to determine the nature, function and potential relationship of various genetic components of the body to disease or body states in animals. Heretofore, the function of a gene has been chiefly examined by the construction of loss-of-function mutations in the gene (i.e., "knock-out" mutations) in an animal (e.g., a transgenic mouse). Such tasks are difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock-out" mutation would produce a lethal phenotype. Moreover, the loss-of-function phenotype cannot be transiently introduced during a particular part of the animal's life cycle or disease state; the "knock-out" mutation is always present. "Antisense knockouts," that is, the selective modulation of expression of a gene by antisense compounds, rather than by direct genetic manipulation, overcomes these limitations (see, for example, Albert et al., *Trends in Pharmacological Sciences*, 1994, 15, 250). In addition, some genes produce a variety of mRNA



transcripts as a result of processes such as alternative splicing; a "knock-out" mutation typically removes all forms of mRNA transcripts produced from such genes and thus cannot be used to examine the biological role of a particular mRNA transcript. Antisense compounds have been systemically administered to rats in order to study the role of the N-methyl-D-aspartate receptor in neuronal death, to mice in order to investigate the biological role of protein kinase C- $\alpha$ , and to rats in order to examine the role of the neuropeptide Y1 receptor in anxiety (Wahlestedt *et al.*, *Nature*, 1993, 363:260; Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1994, 91:11762; and Wahlestedt *et al.*, *Science*, 1993, 259:528, respectively). In instances where complex families of related proteins are being investigated, "antisense knockouts" (i.e., inhibition of a gene by systemic administration of antisense oligonucleotides) may represent the most accurate means for examining a specific member of the family (see, generally, Albert *et al.*, *Trends Pharmacol. Sci.*, 1994, 15:250). By providing compositions and methods for the simple non-parenteral delivery of oligomeric compounds and other nucleic acids, the present invention overcomes these and other shortcomings.

The administration of therapeutic or pharmaceutical compositions comprising the oligomeric compounds of the invention is believed to be within the skill of those in the art. In general, a patient in need of therapy or prophylaxis is administered a composition comprising an oligomeric compound of the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01  $\mu$ g to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution or prevention of the disease state

is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual antisense compounds, and can generally be estimated based on  $EC_{50}$ s found to be effective in *in vitro* and *in vivo* animal models.

In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities of administration of one or more compositions of the invention. A particular treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the composition may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

An optimal dosing schedule is used to deliver a therapeutically effective amount of the oligomeric compound of the invention. The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of oligomer-containing pharmaceutical composition which is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs et al., Chapter 27 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro,

Prophylactic modalities for high risk individuals are also encompassed by the invention. As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., individual or family history or genetic testing, that there is a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. For example, a subject animal could have a personal and/or family medical history that includes frequent occurrences of a particular disease or disorder. As another example, a subject animal could have had such a susceptibility determined by genetic screening according to techniques known in the art (see, e.g., U.S. Congress, Office of Technology Assessment, Chapter 5 *In: Genetic Monitoring and Screening in the Workplace*, OTA-BA-455, U.S. Government Printing Office, Washington, D.C., 1990, pages 75-99). As part of a treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have compared to the effect observed when a

second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

For therapeutic use the oligomeric compound is administered to an animal suffering from a disease modulated  
5 by some protein. It is preferred to administer to patients suspected of suffering from such a disease an amount of oligonucleotide analog that is effective to reduce the symptomology of that disease. One skilled in the art can determine optimum dosages and treatment schedules for such  
10 treatment regimens.

It is preferred that the RNA or DNA portion which is to be modulated be preselected to comprise that portion of DNA or RNA which codes for the protein whose formation or activity is to be modulated. The targeting portion of the composition to  
15 be employed is, thus, selected to be complementary to the preselected portion of DNA or RNA, that is to be an antisense oligomer for that portion.

In accordance with one preferred embodiment of this invention, the compounds of the invention hybridize to HIV  
20 mRNA encoding the tat protein, or to the TAR region of HIV mRNA. In another preferred embodiment, the compounds mimic the secondary structure of the TAR region of HIV mRNA, and by doing so bind the tat protein. Other preferred compounds complementary sequences for herpes, papilloma and other  
25 viruses.

It is generally preferred to administer the therapeutic agents in accordance with this invention internally such as orally, intravenously, or intramuscularly. Other forms of administration, such as transdermally, topically, or  
30 intralesionally may also be useful. Inclusion in suppositories may also be useful. Use of pharmacologically acceptable carriers is also preferred for some embodiments.

This invention is also directed to methods for the selective binding of RNA for research and diagnostic purposes.

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Such selective, strong binding is accomplished by interacting such RNA or DNA with compositions of the invention which are resistant to degradative nucleases and which hybridize more strongly and with greater fidelity than known oligonucleotides  
5 or oligonucleotide analogs.

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20 acceptable carriers is also preferred for some embodiments.

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25 resistant to degradative nucleases and which hybridize more strongly and with greater fidelity than known oligonucleotides or oligonucleotide analogs.

Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred  
30 embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

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**EXAMPLES****General:**

Solvents were dried by distillation:

THF over sodium benzophenone ketyl; acetonitrile and  
5 triethylamine over calcium hydride; and pyridine over barium  
oxide. DBU is distilled under vacuum and then stored over 4 Å  
Linde molecular sieves under argon. PCl<sub>3</sub> is first degassed by  
refluxing for 2 h under argon followed by fractional  
distillation and storage under argon. Water is HPLC grade  
10 obtained from Aldrich Chemical Co. Inc.

**Example 1****Isomerically pure R and S isomers of 4-mercapto-4-methyl-2-pentanol**

R-4-mercapto-4-methyl-2-pentanol and S-4-mercapto-4-  
15 methyl-2-pentanol are synthesized according to the procedure  
of Eliel and Morris-Natschke (Eliel, E.L., Morris-Natschke,  
S., *J. Am. Chem. Soc.* **1984**, *106*, 2937-2942).

**Example 2****Rp precursor, Compound 1**

20 PCl<sub>3</sub> (1.3 mL, 15 mmol) is introduced via a syringe into  
a dry 100-mL round-bottomed flask containing 20 mL of dry THF  
that has been flushed with argon and sealed with a septum. .  
The flask is cooled to -78°C in a dry ice/acetone bath, and a  
solution of  
25 (R)-4-mercapto-4-methyl-2-pentanol (15 mmol) in THF (15 mL)  
containing triethylamine (6.9 mL, 50 mmol) is added via a  
syringe. The reaction mixture is stirred for 30 min at -78°C  
and then warmed to 0°C for 1 hour. The reaction mixture is  
partitioned between CH<sub>2</sub>Cl<sub>2</sub> and saturated NaHCO<sub>3</sub> and washed with  
30 saturated NaCl and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> to give the  
title compound.

**Example 3****Compound 2**

Compound 1 in hexane is treated with morpholine by careful dropwise addition at 0° C. The cold bath is removed, and the mixture is stirred at room temperature for an additional 1 hour. Morpholine hydrochloride is removed by filtration, and Compound 2 is purified by silica gel column chromatography.

**Example 4**

10 **General procedure for the synthesis of monomers used for synthesizing Rp linkages, Structure 3**

To a sample of 2'-deoxy-5'-O-DMT nucleoside (2'-O-deoxy, 5'-O-DMT-6-N-benzoyl adenosine, 2'-O-deoxy, 5'-O-DMT-4-N-benzoyl cytidine, 2'-O-deoxy, 5'-O-DMT-2-N-isobutyl guanosine, 2'-O-deoxy-5'-O-DMT-thymidine or a modified optionally protected 5-O-DMT-nucleoside) 10 mmol in dry CH<sub>2</sub>Cl<sub>2</sub> at -78°C is added 20 mL of a 15 mmol solution of 1H-tetrazole (11 mmol), in THF via syringe. The reaction mixture is stirred at -78°C for 30 min, the cooling bath is removed, and the solution is warmed to room temperature. To this solution is added Compound 2 in THF (11 mmol) dropwise with stirring for 2-4 hours. The sulfurization reagent 3H-1,2-benzodithiole-3-one-1,1-dioxide (2% in CH<sub>3</sub>CN), (Iyer et al., *J. Am. Chem. Soc.* **1990**, 112, 1253) is added with stirring for 1 hour. The solvent is evaporated and the nucleoside oxathiane intermediate is purified by silica gel column chromatography to afford the respective monomeric compound having Structure 3.

**Example 5**

30 **Attachment of thymidine to solid support (5'-HO-T-CPG)**

Thymidine was attached to solid support following a literature procedure (Damha *et al.*, *Nucleic Acids Res.*, **1990**, 18, 3813-3821). To a dry 6 mL Hypovial was added 5'-O-DMT-thymidine (109 mg, 0.2 mmol), CPG with sarcosinyl-succinonyl linker (Brown *et al.*, *J. Chem. Soc. Chem. Comm.* **1989**, 891) (1.0g), 4-DMAP (12 mg, 0.1 mmol), triethylamine (80  $\mu$ L), DEC (384 mg, 2.0 mmol), and anhydrous pyridine (5 mL). The mixture was shaken at room temperature for 24 h. Pentachlorophenol (134 mg, 0.5 mmol) was added, and the mixture was shaken for an additional period of 16 h. The CPG was filtered off and washed successively with pyridine,  $\text{CH}_2\text{Cl}_2$ , and ether. The CPG was treated with reagent grade piperidine (5 mL), and the slurry was shaken for 10 min. The resulting CPG was filtered off, washed successively with  $\text{CH}_2\text{Cl}_2$  and ether, and dried under vacuum. The dried CPG was mixed with equal parts of two solutions of 0.5 M acetic anhydride in THF and 0.5 M 4-DMAP/2,4,6-trimethylpyridine in THF (4 mL each). The slurry was shaken for 2 hours and washed successively with pyridine,  $\text{CH}_2\text{Cl}_2$ , THF, and ether. The loading amount was measured by Trityl Analysis, 37.9 mol/g. Detritylation with 3% trichloroacetic acid in 1,2-dichloroethane afforded the immobilized thymidine.

### Example 6

#### 25 Solid support bound T-Rp-T dimer, Compound 4

To a sintered glass funnel are added 5'-HO-T-CPG (27 mg, 1 mol) and a solution of Structure 3, where the base is thymine, in acetonitrile (0.2 mL, 0.1 M) followed by 30  $\mu$ L of DBU (0.2 mmol) added by syringe. After 15 minutes, the solid support is washed with acetonitrile ( $3 \times 2$  mL), and then Beaucage's reagent (0.2 mL, 0.1M in THF) is added. The solid support on washing with anhydrous  $\text{CH}_3\text{CN}$  gives the title dimer.



**Example 7****T-Rp-T dimer, Compound 5**

Compound 4 is treated with  $\text{NH}_4\text{OH}$  (28%) at  $50^\circ\text{C}$  for 2 h. The solution is evaporated to dryness, and the residue is  
5 dissolved in water (1 mL) and filtered. The resulting crude material which has been cleaved from the solid support is purified and analyzed by HPLC to give Compound 5, a TT dimer having a chiral Rp internucleoside linkage.

**Example 8**10 **Compound 6**

Compound 6 is prepared following the procedures used to prepare Compound 1, Example 2. S-4-mercapto-4-methyl-2-pentanol (15 mmol) is treated with  $\text{PCl}_3$  (15 mmol) to give upon purification Compound 6.

15 **Example 9****Compound 7**

Compound 6 in hexane is treated with morpholine by careful dropwise addition at  $0^\circ\text{C}$ . The cold bath is removed, and the mixture is stirred at room temperature for an  
20 additional 1 hour. Morpholine hydrochloride is removed by filtration, and Compound 7 is purified by silica gel column chromatography.

**Example 10****Monomers used for Sp linkages, Structure 8**

25 Compound 7 is reacted with a 5'-O-DMT nucleoside in the presence of tetrazole followed by addition of sulfur (Beaucage reagent) to give the desired oxathiane phosphorous derivative compound 8. This procedure is illustrated for the Rp isomer

in Example 4 above. Compound 8 is purified by silica gel column chromatography.

#### Example 11

##### 5 Solid support bound T-Sp-T dimer, Compound 9

To a sintered glass funnel are added 5'-HO-T-CPG (Example 5) (27 mg, 1 mmol), a solution of compound 8 in acetonitrile (0.2 mL, 0.1 M), and 30  $\mu$ L of DBU (0.2 mmol, via syringe). After 15 minutes, the solid support is washed with  
10 acetonitrile (3  $\times$  2 mL), and then Beaucage's reagent (0.2 mL, 0.1M in THF) is added. The solid support on standing for 10 minutes followed by washing with anhydrous CH<sub>3</sub>CN gives the title dimer.

#### Example 12

##### 15 T-Sp-T dimer, Compound 10

Compound 9 is treated with NH<sub>4</sub>OH (28%) at 50°C for 2 h. The solution is evaporated to dryness, and the residue is dissolved in water (1 mL) and filtered. Compound 10 is purified and analyzed using HPLC. The four membered thiane  
20 formation facilitates the formation of the product (Compound 10).

#### Example 13

##### 5-methyl-2-(1-methyl-1-thioethyl) cyclohexanol, Compound 11

Compound 11, is obtained from (+)-pulegone, readily  
25 available in enantiomerically pure form following a literature procedure (Lynch et al., *Tetrahedron Lett.*, **1981**, 22, 2855-2888 and Lynch et al., *J. Am. Chem. Soc.*, **1984**, 106, 2943-2948).

#### Example 14

**Compound 12**

Compound 11 and phosphorous trichloride are added in equimolar proportions to  $\text{CH}_2\text{Cl}_2$  containing two equivalents of pyridine at  $-78^\circ\text{C}$ . After stirring for 1 hour, pyridinium  
5 hydrochloride is filtered off, and the solution is concentrated and purified to give Compound 12.

**Example 15****Compound 13**

Compound 12 in hexane is treated with morpholine by  
10 careful dropwise addition at  $0^\circ\text{C}$ . The cold bath is removed, and the mixture is stirred at room temperature for an additional 1 hour. Morpholine hydrochloride is removed by filtration and Compound 13 is purified by silica gel column chromatography.

**Example 16****Chiral monomers used for Rp linkages, Structure 14**

To a selected 2'-deoxy-5-O-DMT-nucleoside (2'-O-deoxy-5'-O-DMT-6-N-benzoyl adenosine, 2'-O-deoxy-5'-O-DMT-4-N-benzoyl cytosine, 2'-O-deoxy-5'-O-DMT-2-N-butyryl guanosine,  
20 2'-O-deoxy-5'-O-DMT-thymidine or modified optionally protected 5-O-DMT-nucleoside) 10 mmol in dry  $\text{CH}_2\text{Cl}_2$  is added 1H tetrazole (11 mmol). Compound 13 (11 mmol) is added dropwise with stirring for 2-4 hours. The resulting intermediate is oxidized with Beaucage reagent as described above for Compound  
25 3. The nucleoside oxathiane intermediate is purified by silica gel column chromatography.

**Example 17**

**General procedure for preparing chiral dimers having Structure 15**

Compound 14 is condensed with a 5'-HO-T-CPG (Example 5), or other solid support bound 5'-OH-nucleoside, using DBU to give a compound having Structure 15 as described above for Compound 4. Dimers having Structure 15 are treated as per the  
5 procedure of Example 7 to cleave the dimer from the CPG and to deblock the phosphorus thereby giving the free deblocked dimer having Structure 15a.

### Example 18

#### Compound 16

10 Starting from (-)-pulegone, commercially available from Fluka, the isomer of (-)-5-methyl-2-(1-methyl-1-thioethyl) cyclohexanol is obtained following literature procedures (Lynch *ibid*). The compound (-)-5-methyl-2-(1-methyl-1-thioethyl) cyclohexanol is treated with  $\text{PCl}_3$  in  $\text{CH}_2\text{Cl}_2$   
15 containing two equivalents of pyridine at  $-78^\circ\text{C}$ . After stirring for 1 hour, pyridinium hydrochloride is filtered off, and the solution is concentrated and purified to give Compound 16.

### Example 19

#### 20 Compound 17

Compound 16 in hexane is treated with morpholine by careful dropwise addition at  $0^\circ\text{C}$ . The cold bath is removed, and the mixture is stirred at room temperature for an additional 1 hour. Morpholine hydrochloride is removed by  
25 filtration, and Compound 17 is purified by silica gel column chromatography.

### Example 20

#### Synthesis of monomers having Structure 18

To a selected 2'-deoxy-5-O-DMT-nucleoside (2'-O-deoxy-  
30 5'-O-DMT-6-N-benzoyl adenosine, 2'-O-deoxy-5'-O-DMT-4-N-

benzoyl cytosine, 2'-O-deoxy-5'-O-DMT-2-N-butyryl guanosine, 2'-O-deoxy-5'-O-DMT-thymidine or modified optionally protected 5-O-DMT-nucleoside) 10 mmol in dry CH<sub>2</sub>Cl<sub>2</sub> is added 1H tetrazole (11 mmol) followed by dropwise addition of Compound 17 (11 mmol) and stirring for 2-4 hours. The sulfurization reagent 3H-1,2-benzodithiole-3-one-1,1-dioxide (2% in CH<sub>3</sub>CN, Iyer *ibid*), is added and stirred for 1 hour. Solvent is evaporated and the crude material is purified by silica gel column chromatography to give Compound 18.

#### 10 Example 21

##### General procedure for preparing Sp dimers using compound 18

Compound 18 is condensed with a 5'-HO-T-CPG (Example 5), or other solid support bound 5'-OH-nucleoside, using DBU to give a compound having Structure 18a as described above for Compound 4. Dimers prepared from Compound 18a are cleaved from the CPG and deblocked thereby giving the free deblocked Sp chiral dimer 18b.

#### Example 22

##### 5c-Methyl-2t [(1-methyl-1-methylamino) ethyl]-cyclohexan-1r-ol

20 19

The title compound is synthesized according to a literature procedure using (+)-pulegone (He *et al.*, *J. Org. Chem.*, **1990**, 55, 2114-2119) by first preparing 5c-Methyl-2t [(1-methyl-1-benzylamino) ethyl]-cyclohexan-1r-ol. This compound is subjected to hydrogenolysis by Pd/H<sub>2</sub> to give the corresponding amino alcohol (removal of benzyl group). The amino alcohol is then treated with 1 equivalent of HCHO followed by NaCNBH<sub>3</sub> reduction to give the title Compound.

#### Example 23

30 Compound 20

Compound 19 and phosphorous trichloride are added in equimolar proportions to  $\text{CH}_2\text{Cl}_2$  containing two equivalents of pyridine at  $-78^\circ\text{C}$ . After stirring for 1 hour, pyridinium hydrochloride is filtered off, and the solution is

5 concentrated and purified to give a chloro-intermediate compound. The chloro-intermediate compound in hexane is treated with morpholine by careful dropwise addition at  $0^\circ\text{C}$ . The cold bath is removed, and the mixture is stirred at room temperature for an additional 1 hour. Morpholine

10 hydrochloride is removed by filtration, and the morpholino compound is purified by silica gel column chromatography.

To a selected 2'-deoxy-5'-O-DMT-nucleoside (2'-O-deoxy-5'-O-DMT-6-N-benzoyl adenosine, 2'-O-deoxy-5'-O-DMT-4-N-benzoyl cytosine, 2'-O-deoxy-5'-O-DMT-2-N-butyryl guanosine,

15 2'-O-deoxy-5'-O-DMT-thymidine or modified optionally protected 5'-O-DMT-nucleoside) 10 mmol in dry  $\text{CH}_2\text{Cl}_2$  is added 1H tetrazole (11 mmol) followed by dropwise addition of the morpholino compound (11 mmol) and stirring for 2-4 hours. The sulfurization reagent 3H-1,2-benzodithiole-3-one-1,1-dioxide

20 (2% in  $\text{CH}_3\text{CN}$ , Iyer *ibid*), is added and stirred for 1 hour. Solvent is evaporated and the nucleoside oxathiane intermediate Compound 20 is purified by silica gel column chromatography.

#### Example 24

#### 25 Compound 21

Compound 20 is condensed with a 5'-HO-T-CPG (Example 5), or other solid support bound 5'-OH-nucleoside, using DBU to give a compound having Structure 21 as described above for Compound 4. A capping step is added to cap the free amine

30 formed.

**Example 25****Generation of Rp Dimer 21a from Compound 21**

Compound 21 is treated with concentrated ammonium hydroxide for 16 hours to give the cleaved deblocked dimer as the Rp isomer and the chiral adjuvant derived products **22** and **23**.

**Example 26****Compound 24**

From the naturally occurring (-)-pulegone (available from Fluka), compound **24** is obtained as a Chiral Adjuvant following a literature procedure (He et al., *Tetrahedron*, **1987**, 43, 4979-4987).

**Example 27****monomer, Compound 25**

Compound **24** is treated with  $\text{PCl}_3$  (1 equivalent) with excess of Hunig base in THF solvent at  $-5^\circ\text{C}$  for 10 minutes. The resulting chloro compound is treated with a selected 2'-deoxy-5'-O-DMT-nucleoside having a free 3'-OH group (2'-O-deoxy-5'-O-DMT-6-N-benzoyl adenosine, 2'-O-deoxy-5'-O-DMT-4-N-benzoyl cytosine, 2'-O-deoxy-5'-O-DMT-2-N-butyryl guanosine, 2'-O-deoxy-5'-O-DMT-thymidine or modified optionally protected 5'-O-DMT-nucleoside). TLC and  $^{13}\text{C}$  NMR analysis is used to reveal the formation of a single diastereomer. The crude material is washed with saturated sodium bicarbonate and dried over anhydrous sodium sulfate. The resulting material is purified either by crystallization or by silica gel column chromatography.

**Example 28****protected dimer, Compound 26**

Purified compound 25 is condensed with a 5'-HO-T-CPG (Example 5), or other solid support bound 5'-OH-nucleoside (such as 2'-O-deoxy-6-N-benzoyl adenosine, 2'-O-deoxy-4-N-benzoyl cytidine, 2'-O-deoxy-2-N-isobutyryl guanosine or other modified optionally protected 5'-OH'-3'-CPG-nucleoside), for 2 hours using tetrazole as the coupling agent. The resultant free amine is capped with acetic anhydride, and the dimer is oxidized with Beaucage reagent to give Compound 26 attached to solid support. Compound 26 is cleaved from the solid support and deprotected by treatment with concentrated ammonium hydroxide (30%, 12 hours). The chiral auxiliary is removed as an isomer of compound 22 or 23 and the oligomer is purified by HPLC. The nucleoside dimer is treated with 80% aqueous acetic acid to remove the 5'-triyl group. The Sp configuration is assigned as described below in the procedures.

**Example 29****Synthesis of Chirally pure 5'-T<sub>Sp</sub>T<sub>Rp</sub>T<sub>Rp</sub>T<sub>Rp</sub>T<sub>Rp</sub>T<sub>Sp</sub>T-3'****20 phosphorothioate heptamer**

50 milligram (2 μmole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage is taken up in a glass reactor, and a toluene solution of 3% dichloroacetic acid (v/v) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile and a 0.2 M solution of Compound 8 (B=T) in acetonitrile (25 fold excess) and a 0.5 M solution of DBU in acetonitrile (200 fold excess) is added and allowed to react at room temperature for 15 minutes. The product is washed with acetonitrile followed by the addition of a 0.2 M solution of Beaucage reagent in acetonitrile with reaction allowed to progress at room temperature for 5 minutes. This sulfurization step is repeated one more time for 5 minutes. The support is washed



with acetonitrile, and then a solution of acetic anhydride/lutidine/ THF (1:1:8), and N-methyl imidazole/ THF is added to cap any unreacted 5'-hydroxyl groups. The product is washed with acetonitrile.

- 5 In the next cycle Compound 3 (B=T) is used as the incoming monomer and the cycle is repeated. This complete cycle is repeated four more times to introduce the Rp linkages. In the final cycle Compound 8 is used as the incoming monomer which introduces the terminal Sp linkage.
- 10 The solid support containing the heptamer is treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature. The aqueous solution is filtered, and concentrated under reduced pressure to give the chirally pure phosphorothioate heptamer.

15 **Example 30**

**Synthesis of Chirally pure 5'-d(G<sub>Sp</sub>A<sub>Rp</sub>C<sub>Sp</sub>T)-3' phosphorothioate tetramer**

- 50 milligram (2  $\mu$ mole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage
- 20 is taken up in a glass reactor, and a toluene solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile, a 0.2 M solution of Compound 8 with B=dC<sup>Bz</sup> in acetonitrile (25 fold excess) and a 0.5 M solution of DBU in
- 25 acetonitrile (250 fold excess) are added, and allowed to react at room temperature for 15 minutes. The product is washed with acetonitrile and a 0.2 M solution of Beaucage reagent in acetonitrile is added and allowed to react at room temperature for 5 minutes. This sulfurization step is repeated one more
- 30 time for 5 minutes. The support is washed with acetonitrile, and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl groups followed by washing with acetonitrile.

In the next cycle Compound 3 ( $B=dA^{Bz}$ ) is used as the incoming monomer and the cycle is repeated. Thus, a 0.2 M solution of Compound 3 with  $B=dA^{Bz}$  in acetonitrile (25 fold excess) and a 0.5 M solution of DBU in acetonitrile (250 fold excess) is added and allowed to react at room temperature for 15 minutes. The product is washed with acetonitrile and a 0.2 M solution of Beaucage reagent in acetonitrile is added and allowed to react at room temperature for 5 minutes. This sulfurization step is repeated one more time for 5 minutes.

10 The support is washed with acetonitrile, a solution of acetic anhydride/ lutidine/THF (1:1:8) and a solution of N-methyl imidazole/THF are added to cap any unreacted 5'-hydroxyl groups. The product is washed with acetonitrile. A solution of 3% dichloroacetic acid in toluene (v/v) is added to

15 deprotect the 5'-hydroxyl groups and the product is washed with acetonitrile.

Compound 8 (0.2 M solution) with  $B=dG^{iBu}$  in acetonitrile (25 fold excess) and a 0.5 M solution of DBU in acetonitrile (200 fold excess) are added and allowed to react at room

20 temperature for 15 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of Beaucage reagent in acetonitrile is added and allowed to react at room temperature for 5 minutes. This sulfurization step is repeated one more time for 5 minutes. The support is washed with acetonitrile

25 and then a solution of acetic anhydride/lutidine/THF (1:1:8) and a solution of N-methyl imidazole/THF are added to cap any unreacted 5'-hydroxyl groups. The product is washed with acetonitrile.

The desired tetramer is deblocked and cleaved from the

30 solid support by treatment with a 30% aqueous solution of ammonium hydroxide for 90 minutes at room temperature followed by heating to 55°C for 12 hours. The aqueous solution is filtered and concentrated under reduced pressure to give the title phosphorothioate tetramer of 5'-dG<sub>Sp</sub>-dA<sub>Rp</sub>dC<sub>Sp</sub>T-3'.

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**Example 31****Oligonucleotide Synthesis: General Procedures**

The oligonucleotides listed in Table 1 are synthesized by following the procedures described above. For generating  
 5 chirally mixed (Rp and Sp) sites, commercial amidites (Perseptive Biosystems) are used and standard synthesis conditions are used.

For introducing Rp linkages with appropriate nucleobases monomers 3, 14 or 20 are used.

10 For introducing Sp linkages with appropriate nucleobases monomers 8, 18 or 25 are used.

The solid support employed is controlled pore glass CPG with sarcosinyl-succinonyl linker (Brown *et al.*, *J. Chem. Soc. Chem. Comm.*, **1989**, 891).

15 The sulfurization reagent employed is 3H-1,2-benzodithiole-3-one-1,1-dioxide (2% in CH<sub>3</sub>CN, Iyer *ibid*).

A solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added mixture to cap any unreacted 5'-hydroxyl group.

20 The preferred reagents have been listed above for the synthesis of chirally pure oligonucleotides. Those skilled in the art will realize that many other reagents and materials are equally amenable to the present invention and that this list is not exclusive.

25

**Table I**

Compound	Sequence	ISIS #/Target
I	GCCCAAGCTG GCATCCGTCA	(ISIS-2302)/Human ICAM-1
II	G <sub>Sp</sub> CCCAAGCTG GCATCCGTC <sub>Sp</sub> A	
III	G <sub>Sp</sub> C <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> A <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> C <sub>Sp</sub> A	
30 IV	TCCGTCATCGCTCCTCAGGG	(ISIS-2503)/Human H-ras
V	T <sub>Sp</sub> CCGTCATCGCTCCTCAGG <sub>Sp</sub> G	
VI	T <sub>Sp</sub> C <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> G <sub>Rp</sub> G <sub>Sp</sub> G	

	<b>VII</b>	GTTCTCGCTGGTGAGTTTCA	(ISIS-3521) / Human PKC- $\alpha$
	<b>VIII</b>	G <sub>Sp</sub> TTCTCGCTGGTGAGTTTC <sub>Sp</sub> A	
	<b>IX</b>	G <sub>Sp</sub> T <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> A <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> T <sub>Rp</sub> T <sub>Rp</sub> C <sub>Sp</sub> A	
	<b>X</b>	TCCCGCCTGTGACATGCATT	(ISIS-5312) / Human C-raf
5	<b>XI</b>	T <sub>Sp</sub> CCCGCCTGTGACATGCAT <sub>Sp</sub> T	
	<b>XII</b>	T <sub>Sp</sub> C <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> A <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Sp</sub> T	
	<b>XIII</b>	GTGCTCATGGTGCACGGTCT	(ISIS-14803) / Human HCV
	<b>XIV</b>	G <sub>Sp</sub> TGCTCATGGTGCACGGTC <sub>Sp</sub> T	
	<b>XV</b>	G <sub>Sp</sub> T <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> C <sub>Rp</sub> G <sub>Rp</sub> G <sub>Rp</sub> T <sub>Rp</sub> C <sub>Sp</sub> T	
10	<b>XVI</b>	TGCATCCCCCAGGCCACCAT	(ISIS-3082) / Murine ICAM-1
	<b>XVII</b>	T <sub>Sp</sub> GCATCCCCCAGGCCACCA <sub>Sp</sub> T	
	<b>XVIII</b>	T <sub>Sp</sub> G <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> T <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> G <sub>Rp</sub> G <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> A <sub>Rp</sub> C <sub>Rp</sub> C <sub>Rp</sub> A <sub>Sp</sub> T	

Table II

SEQ ID NO:	Oligo #	Sequence	ISIS #
15 1	<b>I</b>	GCCCAAGCTG GCATCCGTCA	(ISIS-2302)
2	<b>IV</b>	TCCGTCATCG CTCCTCAGGG	(ISIS-2503)
3	<b>VII</b>	GTTCTCGCTG GTGAGTTTCA	(ISIS-3521)
4	<b>X</b>	TCCCGCCTGT GACATGCATT	(ISIS-5312)
5	<b>XIII</b>	GTGCTCATGG TGCACGGTCT	(ISIS-14803).

20 **Example 32**

**General procedure for the synthesis of gapmer oligomeric compounds using solid phase methodologies 5'-external region**

The 5'-external region is prepared by first attaching a selected nucleoside to a solid support. A modified and  
 25 protected nucleoside is used to incorporate specific nuclease resistant chemistries at the 5'-terminus. An ester or amide attachment through the 3'-hydroxyl is preferred but other motifs described above can be utilized (5', or 2'-attachment). The 5'-hydroxyl (or other hydroxyl) is deprotected and the

next activated nucleoside is reacted with this hydroxyl to  
lengthen the solid support bound compound. Alternate  
chemistries may be employed to prepare nuclease resistant  
internucleoside linkages as discussed above. An alternative  
5 to the monomer approach is to attach a dimer to the solid  
support that has a desired internucleoside linkage. This  
approach allows for the preparation of more complicated  
internucleoside linkages such as MMI.

The incorporation of monomers, dimers or larger  
10 molecules is continued until the 5'-external region is  
completed. The protected hydroxyl group on the last  
nucleoside extending from the solid support is deprotected and  
reacted with a chiral auxiliary that will give a chiral Rp  
internucleoside linkage. Chiral Rp auxiliary compounds are  
15 illustrated in Examples 4, 16 and 23. The procedure of adding  
chiral Rp auxiliary nucleosides is illustrated in examples 29-  
31 above.

After the addition of a desired number and sequence of  
monomers having chiral Rp internucleoside linkages the 5'-  
20 hydroxyl group from the last nucleoside extending from the  
solid support is deblocked. The addition of a nucleoside, a  
nucleosidic dimer or larger molecule to the internal region in  
order to form the 5'-external region is identical to the  
procedure for additions to the 3' external region. After  
25 synthesis of the desired length and sequence the gapmer is  
cleaved from the solid support and deblocked. These two steps  
are routinely performed concurrently as illustrated in  
examples 29-31 above.

### Example 33

#### 30 5'-Building-Blocks

##### Synthesis of 3'-OH nucleoside 27

Modified or unmodified nucleosides, such as Compound 27  
(Figure 8) are synthesized following standard literature

protocols. In addition to methods illustrated above the synthesis of protected 2'-O-methylribonucleosides is described by Sproat et al. and nucleosides with modified bases is described by Connolly in Oligonucleotides and analogs: A practical approach, Ed. F. Eckstein, IRL Press, 1991, p49 and 155, respectively. A variety of protecting groups (Pg<sub>1</sub>) can be utilized for 5'-OH protection. In addition to those previously cited above a comprehensive list is published in Current Protocols in Nucleic Acid Chemistry, Eds. S. L. Beaucage, D. E. Bergstrom, G. D. Glick and R. A. Jones, Wiley, 1999, Chapter 2.3 by H. Seliger. Some of the preferred Pg<sub>1</sub> groups are silanes and photolabile moieties. These groups can be removed under neutral or mild conditions using fluoride treatment or photolysis. A detailed list of such conditions are described in Protective Groups in Organic Synthesis, Eds. T.W. Green and P. Wuts, Wiley, 1999.

#### **Synthesis of backbone modified block 28**

Detailed synthesis of a variety of backbone modified blocks has been published in Comprehensive Natural Products Chemistry: DNA and Aspects of Molecular Biology, Vol.7, Vol. Ed. E. Kool, Eds-in-chief, D. Barton, K. Nakanishi and A. Meth-Cohn, Pergamon Press, 1999, p285. The nucleosidic block can be a dimer, trimer, tetramer or pentamer based on the synthesis requirements. After synthesis of an appropriate block, it is protected with Pg<sub>1</sub> in a manner described above.

#### **Example 34**

##### **Internal pieces**

##### **Synthesis of dNTP( $\alpha$ S) 29**

Natural and synthetic dNTP( $\alpha$ S) are commercially available from many sources. One such source is Amersham Pharmacia Biotech. Appropriate dNTP( $\alpha$ S) are then protected at

3'-OH with  $Pg_2$  using standard chemistry. Some of the preferred  $Pg_2$  groups are esters and phosphates. These groups can be removed under neutral or mild conditions using specific enzymes. A detailed list of such enzymes is described in

5 Protective Groups in Organic Synthesis, Eds. T.W. Green and P. Wuts, Wiley, 1999.

#### Synthesis of App<sub>s</sub>N<sub>p</sub> $g_2$ 31

The synthesis of Compound 31 is performed enzymatically using RNA ligase. App<sub>d</sub>N<sub>p</sub> is one such example that has been

10 described in the literature (Benkovic et al., *Biochemistry*, 1982, 21, 5877). Another example is described in United States Patent Serial No., 5,602,000 issued February 11, 1997, entitled "Method for Enzymatic Synthesis of Oligonucleotides", using T4 RNA ligase method. Following any of the above

15 protocols, an activated compound such as Compound 31 is be prepared. Compound 30 is one of many useful starting materials for the synthesis of Compound 31.

#### Example 35

##### 3'-Building Blocks

#### 20        Synthesis of 32, 33 and 34

Starting with Compound 27, synthesis of dNTP( $\alpha$ S) 32 with a 2'-substituent group can be accomplished following the standard literature procedure described by Eckstein *ibid*. Reaction of 3',5'-diphosphate 34 with ATP in presence of RNA

25 ligase and inorganic pyrophosphate as described in U.S. Patent 5,602,000 cited above will give the desired activated dimer block 33.

#### Synthesis of 35, 36 and 37

Starting with nucleosidic dimer, Compound 28, synthesis

30 of dNTP( $\alpha$ S) Compound 36 having a substituent group at the 2'-position is accomplished following standard literature procedures (see Eckstein *ibid*). Reaction of 3',5'-diphosphate

Compound 35 with ATP in the presence of RNA ligase and inorganic pyrophosphate as described in USP 5,602,000 will give the desired activated dimer block 37.

### Example 36

5 Synthesis of chimeric oligonucleotide with 3' and or 5'-  
modified ends

The overall synthesis of chimeric oligonucleotides having formulas 48-51 is accomplished following a six-step protocol. The first step involves coupling of any 5'-piece as illustrated above and in Figure 8 with an activated middle block as illustrated in figures 10 and 11 in presence of RNA ligase. During this step, the pyrophosphate is converted to a phosphorothioate oligomer having a representative structure as is illustrated in formula 38 with sugar and or base modification or formula 39 with a modified internucleotide linkage. The second step involves removal of the protecting group Pg<sub>2</sub> with an enzyme, such as an esterase or a phosphatase. Reagents are chosen for this step such that the phosphorothioate internucleotide linkage and the 5'-protecting group Pg<sub>1</sub> are unaffected.

The purpose of the second step is to expose the 3'-OH group such that it can be coupled with an appropriate block. Step three is very similar to step one, both require use of RNA ligase and similar reaction conditions. The next step (four) effects removal of the  $Pg_2$  group, exposing the 3'-OH group. Therefore, coupling and removal of  $Pg_2$  groups is continued until a desired length of phosphorothioate oligo is obtained. A further coupling is performed in step 5, thereby attaching the 3'-terminal residue to furnish completed oligomers 46 or 47.

After the last coupling, the protected oligonucleotide is purified using reverse phase column chromatography as described in the literature (Sanghvi et al. in *Manuals of*



Antisense Technology, Eds. G. Hartmann and S. Endres, Kulver Press, 1999, p3). Reverse phase purification will give oligomers having high purity (>95%)..

Removal of the 3' and 5' protecting groups  $Pg_1$  is simultaneously performed during step 6 using mild conditions. One such condition is the use of fluoride ions for the removal of silyl groups. Photolytic removal of protecting groups is also viable at this stage and that has been successfully practiced in the synthesis of oligomers (McGall et al. J. Am. Chem. Soc. 1997, 119, 5081). Alternatively, the terminal ends could be interchanged to provide oligomers having formulas 50 and 51, both bearing a dissimilar nucleosidic unit on the 3' and 5' ends.

### Example 37

#### 15 General procedure for oligonucleotide purification

After the final monomer or blockmer has been added the solid support bound oligonucleotide is deprotected (trityl on) in 1-5 mL 28.0-30% ammonium hydroxide ( $NH_4OH$ ) for approximately 16 hours at 55°C (small scale). For larger scale synthesis of oligonucleotides (20  $\mu$ mol/synthesis) 20 mL of 28.0-30% ammonium hydroxide is used. In general, oligonucleotides are cleaved and deprotected in 5-20 mL 28.0-30%  $NH_4OH$  at 55°C for approximately 16 hours.

Following cleavage and deprotection the crude oligonucleotides are filtered from CPG using Gelman 0.45  $\mu$ m nylon acrodisc syringe filters. Excess  $NH_4OH$  is evaporated away in a Savant AS160 automatic speed vac. The crude yield is measured on a Hewlett Packard 8452A Diode Array Spectrophotometer at 260 nm. Crude samples are then analyzed by mass spectrometry (MS) on a Hewlett Packard electrospray mass spectrometer and by capillary gel electrophoresis (CGE) on a Beckmann P/ACE system 5000. Trityl-on oligonucleotides are purified by reverse phase preparative high performance

liquid chromatography (HPLC). HPLC conditions are as follows: Waters 600E with 991 detector; Waters Delta Pak C4 column (7.8X300mm); Solvent A: 50 mM triethylammonium acetate (TEA-Ac), pH 7.0; B: 100% acetonitrile; 2.5 mL/min flow rate;

5 Gradient: 5% B for first five minutes with linear increase in B to 60% during the next 55 minutes. Larger oligo yields from the larger 20  $\mu$ mol syntheses are purified on larger HPLC columns (Waters Bondapak HC18HA) and the flow rate is increased to 5.0 mL/min. Appropriate fractions are collected

10 and solvent is removed via speed vac. Oligonucleotides are detritylated in 80% acetic acid for approximately 45 minutes and lyophilized again. Free trityl and excess salt are removed by passing detritylated oligonucleotides through Sephadex G-25 (size exclusion chromatography) and collecting

15 appropriate samples with a Pharmacia fraction collector. Concentration of selected fractions gives the purified oligonucleotides which are analyzed for purity by CGE, HPLC (flow rate: 1.5 mL/min; Waters Delta Pak C4 column, 3.9X300mm), and MS. The final yield is determined by

20 spectrophotometer at 260 nm.

### Procedure 1

#### Determination of Configuration of Chiral Thioates

The Rp and Sp configuration of chiral thioates are determined according to the reported procedure (Slim, G.,

25 Gait, M.J., *Nucleic Acids Res.*, **1991** 19, 1183-1188). The Rp isomer elutes in reverse phase column in HPLC as the "fast eluent. (Fraction I)" It is resistant to P1 nuclease but hydrolyzed by snake venom phosphodiesterase. On the other hand, the Sp isomer elutes in HPLC reverse phase column as the

30 "slow" eluent (Fraction II). This stereochemistry gives protection from snake venom phosphodiesterase (SVPD), but this isomer gets hydrolyzed by P1 nuclease.

### Digestion by Snake Venom Phosphodiesterase

An aliquot (2 OD) of each P=S oligonucleotide dimer (both earlier and later eluting peaks by reversed-phase) HPLC is treated for 8 hours at 37°C with snake venom phosphodiesterase (0.1 µg, Boehringer) and calf alkaline phosphatase (6.0 µg, Boehringer) in 0.1 M Tris. HCl (pH 8.5), 0.3 mM dithiothreitol (DTT), 0.3 mM MgCl<sub>2</sub> in a reaction volume of 150 µL. The products are analyzed by reverse phase HPLC. The Rp isomer (the earlier eluting peak) is hydrolyzed while the Sp isomer remains intact.

### Digestion by Nuclease P1

An aliquot of each P=S oligonucleotide dimer (2 ODs) is digested with nuclease P1 (2.0 µg, Boehringer) in distilled water (120 µL) for 1 hour at 37°C. The solution is buffered with 16 µL 0.1 M Tris HCl (pH 8.5) and digested with calf alkaline phosphatase (6.0 µg, Boehringer) for 1 hour at 37°C. The product is analyzed by reverse phase HPLC. In this case, the Sp isomer is degraded while the Rp isomer is resistant to nuclease.

## 20 Procedure 2

### Evaluation of In Vivo Stability of Chimeric Chiral Oligonucleotides

#### Mouse Experiment Procedures

For each oligonucleotide tested, 9 male BALB/c mice (Charles River, Wilmington, MA), weighing about 25 g are used (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923). Following a 1-week acclimation, mice receive a single tail vein injection of oligonucleotide (5 mg/kg) administered in phosphate buffered saline (PBS), pH 7.0. One retro-orbital bleed (either 0.25, 0.5, 2 or 4 lv post dose) and a terminal bleed (either 1, 3, 8 or 24 h post dose) are collected from each group. The terminal bleed (approximately 0.6-0.8 ml) is

collected by cardiac puncture following ketamine/xylazine anesthesia. The blood is transferred to an EDTA-coated collection tube and centrifuged to obtain plasma. At termination, the liver and kidneys are collected from each 5 mouse. Plasma and tissues homogenates are used for analysis for determination of intact oligonucleotide content by CGE. All samples are immediately frozen on dry ice after collection and stored at -80°C until analysis.

### Evaluation of In Vivo Stability of Chimeric Chiral

#### 10 Oligonucleotides

Sub A3  
SEQ ID NO: 5 was used in a comparative study to determine the effect of chiral internucleotide linkages at predetermined positions compared to the same sequence having racemic linkages at each position. The capillary gel electrophoretic 15 analysis indicated the relative nuclease resistance of Chiral 3'-Sp- capped oligomers compared to ISIS 3082 (XVI, uniform 2'-deoxy phosphorothioate). Because of the resistance of Sp linkage to nucleases, Compounds XVII and XVIII are found to be stable in plasma, kidney and liver while XVI (3082) is not. 20 On the other hand, the data from 5',-3'-bis Sp capped oligomers show total exonucleolytic stability in plasma as well as in tissues (liver and kidney). Compounds are stable at various time points such as 1, 3, and 24 hours. The fact that no degradation is detected proved that 5'-exonucleases 25 and 3'-exonuclease are prevalent in tissues and endonucleases are not active. Furthermore, a single chiral linkage (Sp thioate linkage) is sufficient as a gatekeeper against nucleases at the termini.

**Procedure 3****RNase H studies with chimeric Rp and Sp modified oligonucleotides****<sup>32</sup>P Labeling of Oligonucleotides**

5       The oligoribonucleotide (sense strand) is 5'-end labeled with <sup>32</sup>P using [<sup>32</sup>P]ATP, T4 polynucleotide kinase, and standard procedures (Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley, New York (1989)). The labeled RNA is purified by electrophoresis on 12% denaturing PAGE (Sambrook  
10 et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview (1989)). The specific activity of the labeled oligonucleotide is approximately 6000 cpm/fmol.

**Determination of RNase H Cleavage Patterns**

15       Hybridization reactions were prepared in 120 µL of reaction buffer [20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT] containing 750 nM antisense oligonucleotide, 500 nM sense oligoribonucleotide, and 100,000 cpm <sup>32</sup>P-labeled sense oligoribonucleotide. Reactions were heated at  
20 90°C for 5 minutes and 1 unit of Inhibit-ACE is added. Samples were incubated overnight at 37°C degrees. Hybridization reactions were incubated at 37°C with 1.5 x 10.8<sup>-8</sup> mg of *E. coli* RNase H enzyme for initial rate determinations and then quenched at specific time points.  
25 Samples were analyzed by trichloroacetic acid (TCA) assay or by denaturing polyacrylamide gel electrophoresis as previously described [Crooke, S. T., Lemonidis, K. M., Neilson, L., Griffey, R., Lesnik, E. A., and Monia, B. P., Kinetic characteristics of *Escherichia coli* RNase H1: cleavage of  
30 various antisense oligonucleotide-RNA duplexes, *Biochem J*, 312, 599 (1995); Lima, W.F. and Crooke, S. T., *Biochemistry* 36, 390-398, 1997]. In these assays chirally pure Compounds of the type Sp-(Rp)n-Sp showed better Rnase H cleavage activity than diasteromeric mixture Compounds.

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Hybridization reactions were prepared in 120  $\mu$ L of reaction buffer [20 mM Tris-HC (pH 7.5), 20 mM KCl, 10 mM  $MgCl_2$ , 0.1 mM DTT] containing 750 nM antisense oligonucleotide, 500 nM sense oligoribonucleotide, and 100,000 cpm  $^{32}P$ -labeled sense oligoribonucleotide. Reactions were heated at 90°C for 5 min and 1 unit of Inhibit-ACE is added. Samples were incubated overnight at 37°C degrees. Hybridization reactions were incubated at 37°C with  $1.5 \times 10.8^{-8}$  mg of *E. coli* RNase H enzyme for initial rate determinations and then quenched at specific time points. Samples were analyzed by trichloroacetic acid (TCA) assay or by denaturing polyacrylamide gel electrophoresis as previously described (Crooke et al., *Biochem J.*, **1995**, 312, 599; Lima, W.F., and Crooke, S. T., *Biochemistry*, **1997**, 36, 390-398).

#### 15 Procedure 4

##### **Control of H-ras gene expression with chirally defined phosphorothioate oligomers**

H-ras targeted antisense oligonucleotides were tested for the ability to specifically reduce H-ras mRNA in T-24 cells (ATCC, Manassas, Va.). T-24 cells were routinely maintained in complete growth media, DMEM supplemented with 10% fetal calf serum and 100 units per milliliter penicillin and 100 micrograms per milliliter streptomycin (Lifetechnologies, Grand Island, NY) in a humidified incubator at 37°C. For antisense experiments T-24 cells were plated in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of  $2 \times 10^5$  cells per well in complete growth medium and incubated as above. Twenty-four hours after plating the growth media is aspirated and the monolayer is washed once with serum free media (Optimem, Lifetechnologies, Grand Island, NY). Oligonucleotides were formulated in serum free Optimem and Lipofectin (Lifetechnologies, Grand Island, NY) at a constant ratio of 3 micrograms per milliliter Lipofectin per 100

nanomolar oligonucleotide. For oligonucleotide treatment two milliliters of formulated oligonucleotide is added to each well and the cells were incubated for four hours at 37°C. Following incubation the formulated oligonucleotide is

5 aspirated from the monolayer, replaced with growth media, and incubated overnight. Twenty-four hours after treatment total RNA is prepared using RNazol (TEL-TEST, Inc., Friendswood, TX.) following manufactures protocol. RNA is fractionated through 1.2% agarose-formaldehyde gels and transferred to

10 nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) following standard protocols (Sambrook et al. Molecular Cloning a Laboratory Manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989). Nylon membranes were probed for H-ras (Oncogene Research Products,

15 Cambridge, MA) using standard <sup>32</sup>P random priming labeling and hybridization protocols (Sambrook et al. Molecular Cloning a Laboratory Manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989). Following hybridization membranes were imaged using a PhosphorImager (Molecular

20 Dynamics, Sunnyvale, CA) and the images quantified using Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA). Following image analysis membranes were striped of H-ras probe and reprobed for G3PDH (Clontech, Palo Alto, CA) and analyzed as above. H-ras signal is normalized to G3PDH. The mean

25 normalized percent control of triplicates and standard deviation for H-ras signal is calculated. Using this procedure Compounds IV, V and VI are tested. Compounds V and VI show faster efficient reduction of H-ras messages.

#### Procedure 5

#### 30 Determination of ICAM-1 Expression

##### *Oligonucleotide Treatment of HUVECs*

Cells were washed three times with Opti-MEM (Life Technologies, Inc.) prewarmed to 37°C. Oligonucleotides were

premixed with 10g/mL Lipofectin (Life Technologies, Inc.) in Opti-MEM, serially diluted to the desired concentrations, and applied to washed cells. Basal and untreated (no oligonucleotide) control cells were also treated with Lipofectin. Cells  
5 were incubated for 4 hours at 37°C, at which time the medium is removed and replaced with standard growth medium with or without 5 mg/mL TNF-α (R & D Systems). Incubation at 37°C is continued until the indicated times.

***Quantitation of ICAM-1 Protein Expression by***

***10 Fluorescence-activated Cell Sorter***

Cells were removed from plate surfaces by brief trypsinization with 0.25% trypsin in PBS. Trypsin activity is quenched with a solution of 2% bovine serum albumin and 0.2% sodium azide in PBS (+Mg/Ca). Cells were pelleted by  
15 centrifugation (1000 rpm, Beckman GPR centrifuge), resuspended in PBS, and stained with 3 l/10<sup>5</sup> cells of the ICAM-1 specific antibody, CD54-PE (Pharmingen). Antibodies were incubated with the cells for 30 min at 4C in the dark, under gently agitation. Cells were washed by centrifugation procedures and  
20 then resuspended in 0.3 ml of FacsFlow buffer (Becton Dickinson) with 0.5% formaldehyde (Polysciences). Expression of cell surface ICAM-1 is then determined by flow cytometry using a Becton Dickinson FACScan. Percentage of the control ICAM-1 expression is calculated as follows:  
25 [(oligonucleotide-treated ICAM-1 value) - (basal ICAM-1 value)/(non-treated ICAM-1 value) - (basal ICAM-1 value)].  
(Baker et al., *The Journal of Biological Chemistry*, **1997**, 272, 11994-12000).

When ICAM-1 expression is tested with oligomers I, II and  
30 III, it is observed that the ICAM-1 expression data reveal that the oligomers II and III are more efficacious than oligomer I in HUVEC cells. The oligomers are presumably working by a improved nuclease resistance in case of oligomer



II and enhanced RNaseH activity and improved nuclease resistance in the case of oligomer III.

## Procedure 6

## 5-Lipoxygenase Analysis and Assays

5           **A.     Therapeutics**     For therapeutic use, an animal suspected of having a disease characterized by excessive or abnormal supply of 5-lipoxygenase is treated by administering the macromolecule of the invention. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and  
10 repetition rates. Such treatment is generally continued until either a cure is effected or a diminution in the diseased state is achieved. Long term treatment is likely for some diseases.

**B. Research Reagents** The oligonucleotides of this invention will also be useful as research reagents when used to cleave or otherwise modulate 5-lipoxygenase mRNA in crude cell lysates or in partially purified or wholly purified RNA preparations. This application of the invention is accomplished, for example, by lysing cells by standard methods, optimally extracting the RNA and then treating it with a composition at concentrations ranging, for instance, from about 100 to about 500 ng per 10 Mg of total RNA in a buffer consisting, for example, of 50 mM phosphate, pH ranging from about 4-10 at a temperature from about 30° to about 50°

25 C. The cleaved 5-lipoxygenase RNA can be analyzed by agarose gel electrophoresis and hybridization with radiolabeled DNA probes or by other standard methods.

C. **Diagnostics** The oligonucleotides of the invention will also be useful in diagnostic applications, particularly for the determination of the expression of specific mRNA species in various tissues or the expression of abnormal or mutant RNA species. In this example, while the macromolecules

target a abnormal mRNA by being designed complementary to the abnormal sequence, they would not hybridize to normal mRNA.

Tissue samples can be homogenized, and RNA extracted by standard methods. The crude homogenate or extract can be  
5 treated for example to effect cleavage of the target RNA. The product can then be hybridized to a solid support which contains a bound oligonucleotide complementary to a region on the 5' side of the cleavage site. Both the normal and abnormal 5' region of the mRNA would bind to the solid  
10 support. The 3' region of the abnormal RNA, which is cleaved, would not be bound to the support and therefore would be separated from the normal mRNA.

Targeted mRNA species for modulation relates to 5-lipoxygenase; however, persons of ordinary skill in the art  
15 will appreciate that the present invention is not so limited and it is generally applicable. The inhibition or modulation of production of the enzyme 5-lipoxygenase is expected to have significant therapeutic benefits in the treatment of disease. In order to assess the effectiveness of the compositions, an  
20 assay or series of assays is required.

**D. In Vitro Assays** The cellular assays for 5-lipoxygenase preferably use the human promyelocytic leukemia cell line HL-60. These cells can be induced to differentiate into either a monocyte like cell or neutrophil like cell by  
25 various known agents. Treatment of the cells with 1.3% dimethyl sulfoxide, DMSO, is known to promote differentiation of the cells into neutrophils. It has now been found that basal HL-60 cells do not synthesize detectable levels of 5-lipoxygenase protein or secrete leukotrienes (a downstream  
30 product of 5-lipoxygenase). Differentiation of the cells with DMSO causes an appearance of 5-lipoxygenase protein and leukotriene biosynthesis 48 hours after addition of DMSO. Thus induction of 5-lipoxygenase protein synthesis can be

utilized as a test system for analysis of oligonucleotides which interfere with 5-lipoxygenase synthesis in these cells.

A second test system for oligonucleotides makes use of the fact that 5-lipoxygenase is a "suicide" enzyme in that it inactivates itself upon reacting with substrate. Treatment of differentiated HL-60 or other cells expressing 5 lipoxygenase, with 10  $\mu$ M A23187, a calcium ionophore, promotes translocation of 5-lipoxygenase from the cytosol to the membrane with subsequent activation of the enzyme. Following activation and several rounds of catalysis, the enzyme becomes catalytically inactive. Thus, treatment of the cells with calcium ionophore inactivates endogenous 5-lipoxygenase. It takes the cells approximately 24 hours to recover from A23187 treatment as measured by their ability to synthesize leukotriene B<sub>4</sub>. Macromolecules directed against 5-lipoxygenase can be tested for activity in two HL-60 model systems using the following quantitative assays. The assays are described from the most direct measurement of inhibition of 5-lipoxygenase protein synthesis in intact cells to more downstream events such as measurement of 5-lipoxygenase activity in intact cells.

A direct effect which oligonucleotides can exert on intact cells and which can be easily be quantitated is specific inhibition of 5-lipoxygenase protein synthesis. To perform this technique, cells can be labeled with <sup>35</sup>S-methionine (50  $\mu$ Ci/mL) for 2 hours at 37° C to label newly synthesized protein. Cells are extracted to solubilize total cellular proteins and 5-lipoxygenase is immunoprecipitated with 5-lipoxygenase antibody followed by elution from protein A Sepharose beads. The immunoprecipitated proteins are resolved by SDS-polyacrylamide gel electrophoresis and exposed for autoradiography. The amount of immunoprecipitated 5-lipoxygenase is quantitated by scanning densitometry.

A predicted result from these experiments would be as follows. The amount of 5-lipoxygenase protein immuno-

precipitated from control cells would be normalized to 100%. Treatment of the cells with 1  $\mu$ M, 10  $\mu$ M, and 30  $\mu$ M of the macromolecules of the invention for 48 hours would reduce immunoprecipitated 5-lipoxygenase by 5%, 25% and 75% of control, respectively.

Measurement of 5-lipoxygenase enzyme activity in cellular homogenates could also be used to quantitate the amount of enzyme present which is capable of synthesizing leukotrienes. A radiometric assay has now been developed for quantitating 5-lipoxygenase enzyme activity in cell homogenates using reverse phase HPLC. Cells are broken by sonication in a buffer containing protease inhibitors and EDTA. The cell homogenate is centrifuged at 10,000 x g for 30 min and the supernatants analyzed for 5-lipoxygenase activity. Cytosolic proteins are incubated with 10  $\mu$ M  $^{14}$ C-arachidonic acid, 2mM ATP, 50  $\mu$ M free calcium, 100  $\mu$ g/mL phosphatidylcholine, and 50 mM bis-Tris buffer, pH 7.0, for 5 min at 37° C. The reactions are quenched by the addition of an equal volume of acetone and the fatty acids extracted with ethyl acetate. The substrate and reaction products are separated by reverse phase HPLC on a Novapak C18 column (Waters Inc., Millford, MA). Radioactive peaks are detected by a Beckman model 171 radiochromatography detector. The amount of arachidonic acid converted into di-HETE's and mono-HETE's is used as a measure of 5-lipoxygenase activity.

A predicted result for treatment of DMSO differentiated HL-60 cells for 72 hours with effective the macromolecules of the invention at 1  $\mu$ M, 10  $\mu$ M, and 30  $\mu$ M would be as follows. Control cells oxidize 200 pmol arachidonic acid/ 5 min/  $10^6$  cells. Cells treated with 1  $\mu$ M, 10  $\mu$ M, and 30  $\mu$ M of an effective oligonucleotide would oxidize 195 pmol, 140 pmol, and 60 pmol of arachidonic acid/ 5 min/  $10^6$  cells respectively.

5 A quantitative competitive enzyme linked immunosorbant assay (ELISA) for the measurement of total 5-lipoxygenase protein in cells has been developed. Human 5-lipoxygenase expressed in *E. coli* and purified by extraction, Q-Sepharose, hydroxyapatite, and reverse phase HPLC is used as a standard and as the primary antigen to coat microtiter plates. 25 ng of purified 5-lipoxygenase is bound to the microtiter plates overnight at 4° C. The wells are blocked for 90 min with 5% goat serum diluted in 20 mM Tris·HCL buffer, pH 7.4, in the presence of 150 mM NaCl (TBS). Cell extracts (0.2% Triton X-100, 12,000 x g for 30 min.) or purified 5-lipoxygenase were incubated with a 1:4000 dilution of 5-lipoxygenase polyclonal antibody in a total volume of 100 µL in the microtiter wells for 90 min. The antibodies are prepared by immunizing rabbits with purified human recombinant 5-lipoxygenase. The wells are washed with TBS containing 0.05% tween 20 (TBST), then incubated with 100 µL of a 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA) for 60 min at 25°C. The wells are washed with TBST and the amount of peroxidase labeled second antibody determined by development with tetramethylbenzidine.

Predicted results from such an assay using a 30 mer oligonucleotide at 1 µM, 10 µM, and 30 µM would be 30 ng, 18 ng and 5 ng of 5-lipoxygenase per 10<sup>6</sup> cells, respectively with untreated cells containing about 34 ng 5-lipoxygenase.

A net effect of inhibition of 5-lipoxygenase biosynthesis is a diminution in the quantities of leukotrienes released from stimulated cells. DMSO-differentiated HL-60 cells release leukotriene B4 upon stimulation with the calcium ionophore A23187. Leukotriene B4 released into the cell medium can be quantitated by radioimmunoassay using commercially available diagnostic kits (New England Nuclear, Boston, MA). Leukotriene B4 production can be detected in HL-60 cells 48 hours following addition of DMSO to differentiate

the cells into a neutrophil-like cell. Cells ( $2 \times 10^5$  cells/mL) will be treated with increasing concentrations of the macromolecule for 48-72 hours in the presence of 1.3% DMSO. The cells are washed and resuspended at a concentration of  $2 \times 10^6$  cell/mL in Dulbecco's phosphate buffered saline containing 1% delipidated bovine serum albumin. Cells are stimulated with 10  $\mu$ M calcium ionophore A23187 for 15 min and the quantity of LTB<sub>4</sub> produced from  $5 \times 10^5$  cell determined by radioimmunoassay as described by the manufacturer.

Using this assay the following results would likely be obtained with an oligonucleotide directed to the 5-LO mRNA. Cells will be treated for 72 hours with either 1  $\mu$ M, 10  $\mu$ M or 30  $\mu$ M of the macromolecule in the presence of 1.3% DMSO. The quantity of LTB<sub>4</sub> produced from  $5 \times 10^5$  cells would be expected to be about 75 pg, 50 pg, and 35 pg, respectively with untreated differentiated cells producing 75 pg LTB<sub>4</sub>.

**E. In Vivo Assay** Inhibition of the production of 5-lipoxygenase in the mouse can be demonstrated in accordance with the following protocol. Topical application of arachidonic acid results in the rapid production of leukotriene B<sub>4</sub>, leukotriene C<sub>4</sub> and prostaglandin E<sub>2</sub> in the skin followed by edema and cellular infiltration. Certain inhibitors of 5-lipoxygenase have been known to exhibit activity in this assay. For the assay, 2 mg of arachidonic acid is applied to a mouse ear with the contralateral ear serving as a control. The polymorphonuclear cell infiltrate is assayed by myeloperoxidase activity in homogenates taken from a biopsy 1 hour following the administration of arachidonic acid. The edematous response is quantitated by measurement of ear thickness and wet weight of a punch biopsy. Measurement of leukotriene B<sub>4</sub> produced in biopsy specimens is performed as a direct measurement of 5-lipoxygenase activity in the tissue. Oligonucleotides will be applied topically to both ears 12 to 24 hours prior to administration of arachidonic acid to allow

optimal activity of the compounds. Both ears are pretreated for 24 hours with either 0.1  $\mu\text{mol}$ , 0.3  $\mu\text{mol}$ , or 1.0  $\mu\text{mol}$  of the macromolecule prior to challenge with arachidonic acid. Values are expressed as the mean for three animals per concentration. Inhibition of polymorphonuclear cell infiltration for 0.1  $\mu\text{mol}$ , 0.3  $\mu\text{mol}$ , and 1  $\mu\text{mol}$  is expected to be about 10%, 75% and 92% of control activity, respectively. Inhibition of edema is expected to be about 3%, 58% and 90%, respectively while inhibition of leukotriene B<sub>4</sub> production would be expected to be about 15%, 79% and 99%, respectively.

It is intended that each of the patents, applications, printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

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